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Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are widely-distributed stimuli of cellular growth and functions, which are generated enzymatically from membrane precursors in activated normal cells and at much higher levels from many types of cancers. A subfamily of G protein-coupled receptors encoded by endothelial differentiation genes (Edg Rs) bind and transduce cellular signals from LPA (Edg-2, -4, -7) and S1P (Edg-1, -3, -5, -6, and –8). Human breast cancer cells (BCCs) express principally Edg-3, less Edg-4, very low levels of Edg-2 and Edg-5, and no Edg-1, -6 or –8. BCCS have much higher levels of Edg-3 S1P Rs than normal human breast epithelial cells. These Edg Rs mediate proliferation of BCCs directly by signaling growth-related immediate-early genes, through a distinctive set of transcription factors, and indirectly by increasing BCC secretion of several autocrine protein growth factors. Delivery of LPA to BCCs by specific plasma protein carriers, such as gelsolin, and novel mechanisms for regulation of expression of Edg-3 Rs also may distinguish mechanisms of action of S1P and LPA in BCCs from those in normal breast epithelial cells. Detection of elevated levels of Edg-3 Rs and splice variants of Edg-3 Rs may become a diagnostic index of breast cancer and Edg R-directed antagonists may suppress growth and dissemination of breast cancers.

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INTRODUCTION:

A subfamily of at least eight structurally homologous G protein-coupled receptors for the growth factors lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are encoded by endothelial differentiation genes (Edg Rs). Edg-1, -3, -5, -6 and –8 Rs are specific for S1P and Edg-2, -4 and –7 are specific for LPA. Edg Rs transduce two distinct types of cellular responses to LPA and S1P. The **first** are growth-related and include direct nuclear signaling of immediate-early genes by induction of a distinctive set of transcription factors and indirect signaling through increases in both generation of autocrine protein growth factors and expression of their receptors. The **second** are growth-independent and encompass a cluster of cytoskeleton-dependent functions, including adhesion, migration, contraction and secretion. Preliminary studies showed that human breast cancer cells (BCCs) of several cultured lines express high levels of some Edg Rs, which transduce LPA and S1P stimulation of proliferation by: i. activation of serum-response element (SRE)-driven transcription of growth genes, ii. enhancement of BCC generation of insulin-like growth factor II (IGF-II), and iii. increased BCC-surface expression of type I receptors for IGF-II (IGF-I-Rs) and heparin-binding epidermal growth factor-like growth factor (HB-EGF), which augments proliferation by interacting with both EGF Rs and proteoglycans of cells and tissues. The results of studies in the second year have addressed the remaining aspects of **Task 1** of the Statement of Work, including completion of analyses of the effects of 1, 25-dihydroxy-vitamin D3 and homologous ligand on the expression of Edg Rs and their signaling pathways by BCCs. In order to assess the involvement of individual Edg Rs in direct stimulation of SRE by defined ligands in BCCs, to complete **Task 2**, new methods for quantifying biochemical pathways and novel functional anti-Edg R monoclonal antibodies have been developed and applied in several lines of human BCC. **Tasks 3 and 4** have not yet been started because optimal conditions had to be established first for Edg R stimulation of components of the IGF-II/IGF-I R axis and HB-EGF/EGF R axis, and more sensitive respective methods developed for assessing changes in each component.

BODY:

a) **Hypothesis and specific aims-** The central hypothesis is that S1P and LPA stimulate proliferation and malignant functions of BCCs by several distinct Edg R-dependent mechanisms, including direct transcriptional activation of immediate-early growth-related genes with controlling SRE sequences and upregulation of autocrine protein growth factor circuits. Further, it is suggested that functionally-significant differences in these pathways will be found which distinguish between BCCs and non-malignant breast epithelial cells.

b) **Task 1-** Delineate determinants of expression of Edg Rs by human cultured breast cancer cells (BCCs)

Initial studies of estrogen, the LPA and S1P ligands and 1, 25-dihydroxy-vitamin D3 (VD3) indicated that LPA, S1P and VD3 all were capable of regulating expression of the principal Edg Rs of BCCs except for estrogen, for which no effects were observed at physiological concentrations. Edg R expression was quantified by TaqMan real-time PCR and Western blots with mouse monoclonal anti-Edg R peptide antibodies. Quantitative PCR has substantially improved our ability to determine precisely the levels of mRNA encoding each

Edg R. For example, in the MDA-MB-453 line of human BCCs, Edg-3 seemed dominant quantitatively by conventional RT-PCR, but nearly equal levels of Edg-2, -4, and –5 also were detected. With real-time quantitative PCR, the respective mean representation of Edg-1, -2, -3, -4 and –5 was: 0, 1.4, 60, 32, and 2.2. At 1 nM to 1 uM, LPA downregulated Edg-2 Rs and less effectively Edg-4 Rs by respective mean maxima of up to 83% and 36% after 30 min at 37°C. Similarly, 1 nM to 1 uM S1P reduced expression of Edg-3 and Edg-5 Rs, respectively, by mean maxima of 59% and 90% after 30 min at 37°C. LPA had no effect on expression of Edg-3 and Edg-5 Rs, and S1P had no effect on expression of Edg-2 and Edg-4 Rs by MDA-MB-453 BCCs. The characteristics of ligand-independent alterations in expression of Edg Rs by VD3 were elucidated by a detailed series of experiments. A 24 h exposure to VD3 suppressed Edg R-encoding mRNA with V3 concentration-dependence, similar effects on Edg-2, -3 and –5, but no suppression of Edg-4 R mRNA (Fig. 1). Thus VD3 pretreatment effectively shifts the predominant sensitivity of BCCs from S1P to LPA. The increases in $[Ca^{2+}]i$ induced in MDA-MB-453 BCCs by S1P and LPA also were suppressed by pretreatment with VD3, with ligand concentration-dependence and increased suppression in relation to the time of pretreatment (Fig. 2). The magnitude of suppression of $[Ca^{2+}]i$ responses to S1P by VD3 were far greater than that of equivalent responses to LPA, as expected by the greater reduction in Edg-3 than Edg-4 Rs. A report of these results has been submitted for publication.

c) Task 2- Characterize mechanisms of Edg R enhancement of SRE-regulated transcription in human cultured BCCs

These studies proved more challenging than expected and required development of two additional capabilities, the first of which was generation of activating monoclonal anti-Edg-3 and -4 R antibodies and the second was luciferase reporter constructs of two sub-components of the SRE element. The basic observation is highly reproducible, with striking enhancement of the SRE report by LPA and S1P (Fig. 3). S1P exhibited 100-fold higher potency than LPA. VD3 suppressed SRE reports evoked by each stimulus, with far less difference than that observed for $[Ca^{2+}]i$ responses. These results raised questions about the Edg R-transduction of SRE responses to LPA and S1P. Newly-developed anti-Edg-3 and anti-Edg-4 R antibodies with agonist activity gave the same results as the primary LPA and S1P stimuli. The use of a new reporter construct composed of the Elk-1 substituent of SRE in a luciferase reporter plasmid demonstrated that LPA and S1P signals were transduced by their respective Edg Rs, as equivalent reports were evoked by S1P and anti-Edg-3 antibody, and by LPA and anti-Edg-4 antibody. In this system, VD3 suppressed luciferase responses to S1P, but not to LPA, as expected from the differential inhibition of expression of Edg-3 but not Edg-4 Rs.

d) Task 3- Elucidate the pathways by which Edg Rs transduce LPL enhancement of both secretion of IGF-II and expression of IGF-I-Rs

These studies are just now beginning and will use the newly-developed agonist antibodies as well as LPA and S1P to verify the involvement of Edg R transduction in the observed responses.

KEY RESEARCH ACCOMPLISHMENTS:

- Development of anti-Edg-3 R and anti-Edg-4 R monoclonal antibodies with functional effects on BCCs.
- Characterization and application of biochemical assays of Edg-3 R and Edg-4 R signaling in BCCs.
- Finding that BCC migration through Matrigel on a micropore filter, as a model of trans-basement membrane passage into tissues, is altered by LPA and S1P in relation to Edg R expression.
- Verification that 1, 25-dihydroxy-vitamin D3 selectively suppresses expression of Edg-3 Rs and to a lesser extent Edg-2 and Edg-5 Rs, but not Edg-4 Rs in BCCs by transcriptional mechanisms and to an extent that modifies functional responses.

REPORTABLE OUTCOMES:

Publications-

i. Goetzl, E.J., Lee, H., Azuma, T., Stossel, T.P., Turck, C.W., and Karliner, J.S. Gelsolin binding and cellular presentation of lysophosphatidic acid. J. Biol. Chem. 275: 14573-14578, 2000.

ii. Goetzl, E.J., An, S., and Lee, H. Lysophospholipid growth factor receptors. Cytokine Reference Database. Academic Press, pp. 2255-2260, 2000.

iii. Goetzl, E.J., Lee, H., and Tigyi, G.J. Lysophospholipid growth factors. Cytokine Reference Database. Academic Press, pp. 1407-1418, 2000.

iv. Goetzl, E.J. Pleiotypic mechanisms of cellular responses to biologically active lysophospholipids. Prostaglandins and other Lipid Mediators 64: 11-20, 2001.

CONCLUSIONS: The results to date clearly show that Edg-3 Rs are upregulated and predominate on BCCs, compared to normal human breast epithelial cells, and therefore that S1P is the major LPL signal for BCCs. The involvement of both direct and indirect signaling from Edg-3 Rs in the biochemical and biological responses of BCCs to S1P also has been confirmed. Thus Edg-3 may be a marker for breast cancer and pharmacological antagonists specific for Edg-3 Rs may have therapeutic benefits in some breast cancer patients. These results also clearly distinguish the abnormalities in the Edg Rs of BCCs from those of ovarian cancer cells, where the predominant axis is LPA and Edg-4 Rs.

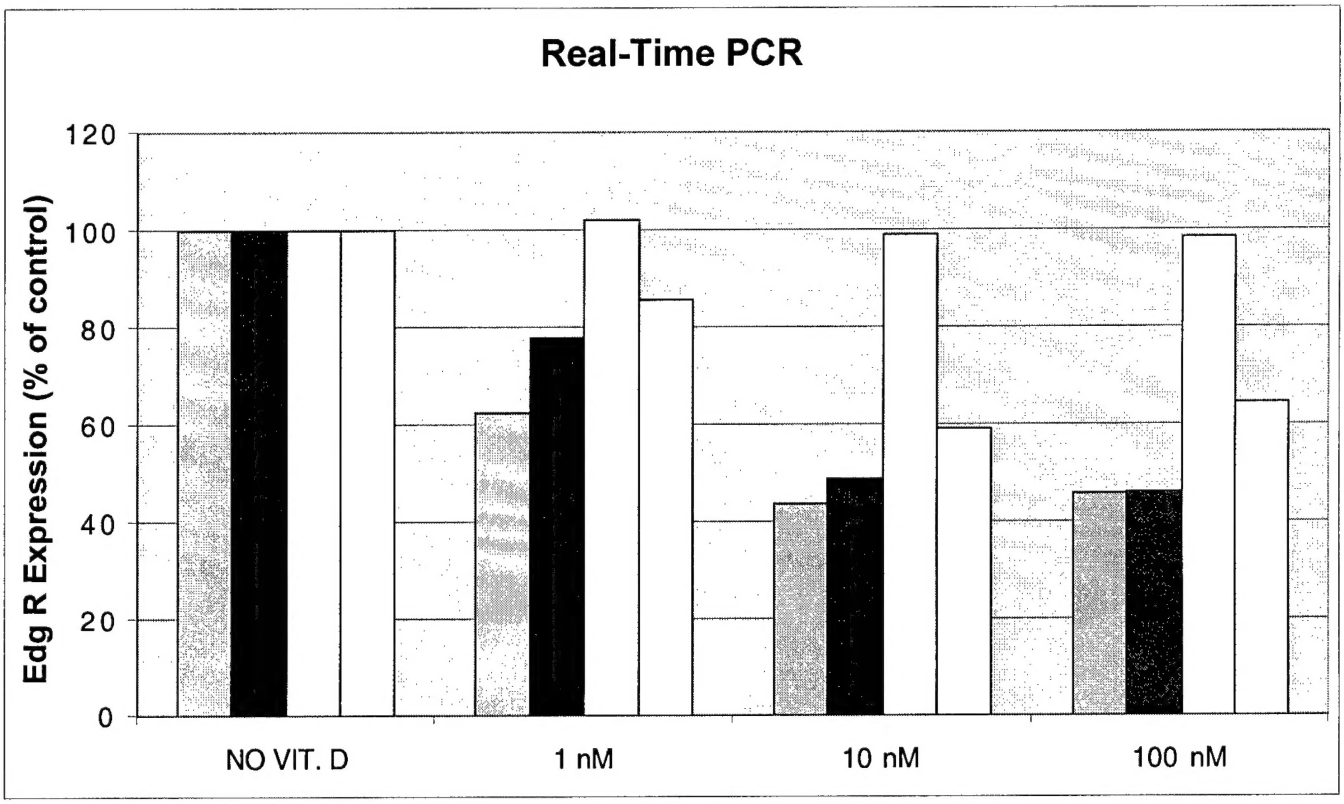

Figure 1. Relative Quantification of Edg R Expression Using the Comparative C_T Method. Edg R expression in MDA-MB-453 BCCs treated with 1-100 nM 1,25- dihydroxyvitamin D_3 for 24 hours was normalized to Gus and is shown as a percentage of the control (calibrator – untreated sample). Each bar represents the mean value from three separate RT PCR amplifications with error bars (too small to be visible) representing standard deviation calculated according to ref.

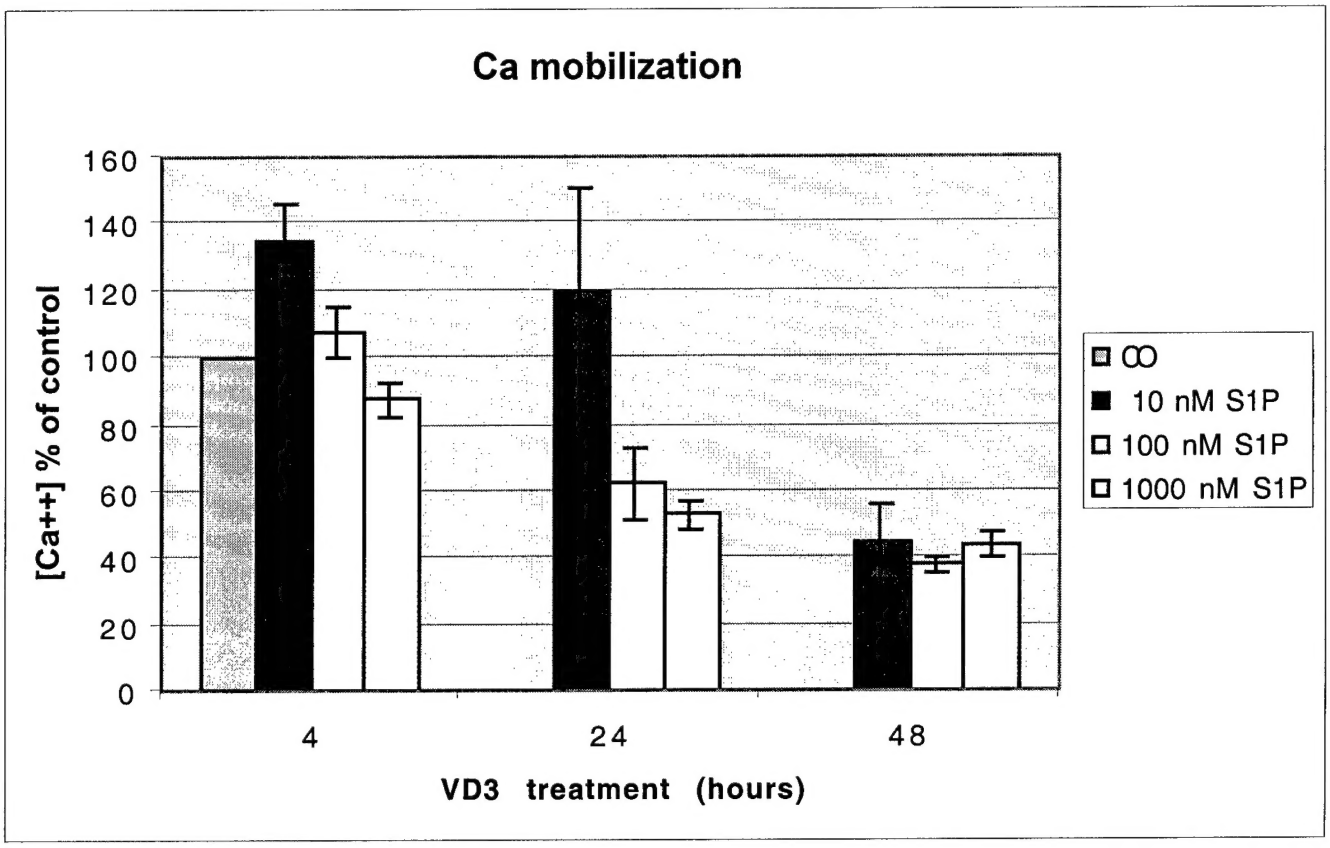

Figure 2. Effect of VD3 on S1P-induced Ca^{2+} response. MDA-MB-453 cells were pretreated with 1nM VD3 for 4, 24 and 48 h and S1P (10-1000 nM)-induced Ca^{2+} mobilization was assayed. Results are shown as a percentage of the values obtained in vehicle-treated control cells.

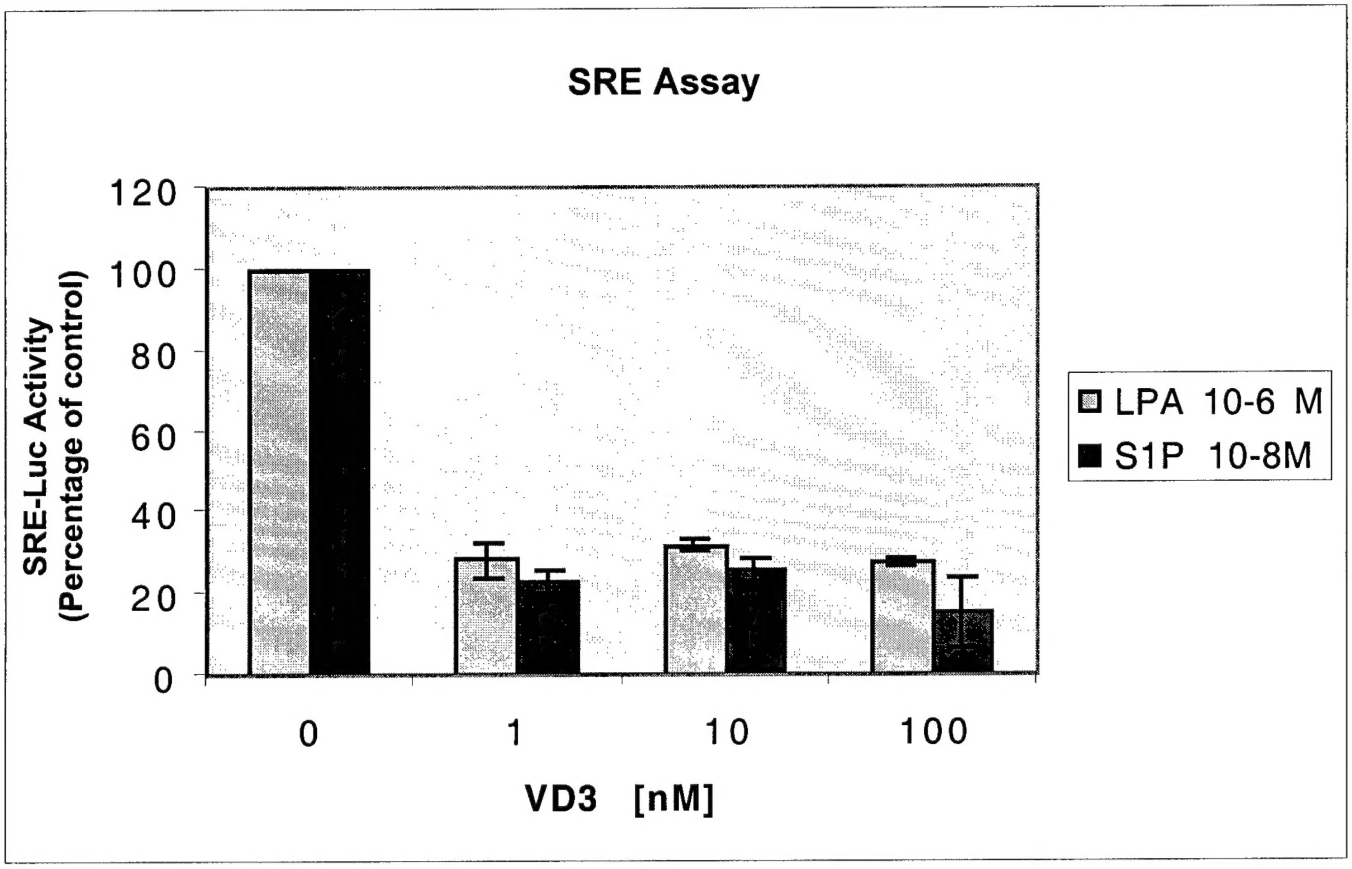

Figure 3. VD3 suppresses LPL-induced nuclear signaling in MDA-MB-453 BCCs. BCCs were transiently co-transfected with pSRE-Luc and pCMV-Renilla vectors and treated with increasing concentrations of VD3 (1-100 nM) for 24 h. The cells were then incubated with LPLs, at the indicated concentrations, for 4h and lucifearse activity was measured. Firefly luciferase values were corrected by Renilla luciferase signals and expressed as a fold increase over LPL-untreated control in corresponding samples. The values for VD3 untreated samples were 6.45±1.17 for media control; 112.43±7.76 for LPA-, 84.1±11.88 for S1P-treated cells. Data are shown as a percentage of VD3 untreated (vehicle treated cells) control values. Results are means of three studies performed in triplicate, with standard error bars.

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Gelsolin Binding and Cellular Presentation of Lysophosphatidic Acid*

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Lysophosphatidic acid (LPA) in biological fluids binds to serum albumin and other proteins that enhance its effects on cellular functions. The actin-severing protein gelsolin binds LPA with an affinity (K_d = 6 nM) similar to that of the G protein-coupled LPA receptors encoded by endothelial differentiation genes 2, 4, and 7 (Edg-2, -4, and -7 receptors) and greater than that of serum albumin (K_d = 360 nM). At concentrations of 10% or less of that in plasma, which are observed in fluids of injured tissues, purified and recombinant gelsolin augment LPA stimulation of nuclear signals and protein synthesis in rat cardiac myocytes (RCMs) that express Edg-2 and -4 receptors. At concentrations of 20% or more of that in plasma, gelsolin suppresses LPA stimulation of RCMs. The lack of effect of gelsolin on RCM responses to monoclonal anti-Edg-4 receptor antibody plus a phorbol ester without LPA attests to its specificity for LPA delivery and the absence of post-receptor effects. Inhibition of gelsolin binding and cellular delivery of LPA by L-α-phosphatidylinositol-4,5-bisphosphate (PIP2) and peptides constituting the two PIP2 binding domains of gelsolin suggests competition between LPA and PIP2 for the same sites. Thus, delivery of LPA to RCMs is affinity-coupled to Edg receptors by gelsolin in a PIP2-regulated process.

Lysophosphatidic acid (LPA)[1] and sphingosine 1-phosphate (S1P) are highly active cellular growth factors that are generated enzymatically from cellular membrane precursors and secreted by activated platelets, leukocytes, epithelial cells, and some types of tumors (1–3). Extracellular LPA and S1P are bound extensively by serum albumin, which also provides for their delivery to cellular receptors (4–6). In one of the earliest studies designed to identify cellular receptors for LPA, the extension of a receptor affinity-labeling method to serum proteins in one experiment detected interactions of LPA with proteins of 15, 28, and 80 or more kDa as well as serum albumin (7). However, these proteins were not resolved or characterized further, and no studies ever were conducted of equilibrium binding of LPA to serum albumin or any other protein.

Extensive investigations have determined the fatty acid and phospholipid binding properties of serum albumin, which include equilibrium dissociation constants as low as 10 nM for some fatty acids and as high as 300 to 1,000 nM for larger and more polar fatty acids with limited access to the high affinity pockets (8–10). However, these studies using classical techniques did not include analyses of binding of LPA to serum albumin, in part due to the high levels of unspecific binding attributable to the amphipathic nature of LPA. It was discovered recently that one or more proteins secreted into serum-free medium of cultured rat cardiac myocytes (RCMs) delivered LPA, but not S1P, to RCMs and some other cells more effectively than fatty acid-free bovine serum albumin (faf-BSA) (11). LPA regulates the actin-binding and -severing activities of gelsolin, an approximately 85-kDa cytosolic constituent of myocytes and a myocyte-derived plasma protein, by mechanisms similar to those of L-α-phosphatidylinositol-4,5-bisphosphate (PIP2), which binds to two distinct sites of gelsolin (12–14). This raised the possibility that gelsolin, which normally is present in plasma and extracellular fluids at a concentration of 100 to 250 μg/ml, may be one of the RCM-derived proteins capable of binding LPA and then either sequestering it or delivering it to cellular receptors. A sub-family of G protein-coupled receptors encoded by endothelial differentiation genes (EdgRs) recently has been shown to bind and transduce signals from several lysolipid phosphate mediators (15–22). At nanomolar concentrations of ligands, Edg-2, -4, and -7 receptors are specific for LPA and Edg-1, -3, and -5 receptors for S1P.

This study was designed to quantify equilibrium binding of LPA to gelsolin and faf-BSA, characterize the gelsolin binding sites for LPA in relation to those for PIP2, and investigate the capacity of gelsolin to deliver LPA to RCMs and elicit RCM nuclear, biochemical, and functional responses.

EXPERIMENTAL PROCEDURES

Chemical Reagents and Antibodies—Sources of chemicals were as follows. Purified human plasma gelsolin was from Sigma, human recombinant gelsolin was from Biogen Corp., Cambridge, MA, recombinant *Clostridium botulinum* ADP-ribosyltransferase (C3 exoenzyme), was from List Biological Laboratories, Inc., Campbell, CA, pRL-CMV *Renilla* luciferase vector was from Promega, Inc., Madison, WI, and FuGENE 6 transfection reagent was from Roche Molecular Biochemicals Corp. Recombinant gelsolin has the proper pairing of sulfhydryl groups to ensure native conformation and full biological activity, as described previously (23, 24) and reproven recently (Biogen Corp.). The villin 133-YDVQRLLHVKGKRNV-147 (25), gelsolin 135-KSGLKYKK-GGVASGF-149 (13) (gel P1), gelsolin 150-KHVVPNEVVVQRLFQVKG-RR-169 (14) (gel P2), and control random gelsolin 150-VNPEVLRV-

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[1] The abbreviations used are: LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; PIP2, L-α-phosphatidylinositol 4,5-bisphosphate; EdgR, endothelial differentiation gene-encoded G protein-coupled receptor; RCM, rat cardiac myocyte; DMEM*, Dulbecco's modified minimal Eagle's medium with penicillin and streptomycin; SRE, serum response element; faf-BSA, fatty acid-free bovine serum albumin; PMA, phorbol myristate acetate.

FKQHVVRGVRKQ-169 substituent peptides were synthesized using a solid-phase system (Model 433 Peptide Synthesizer, Perkin-Elmer) with fluorenylmethoxycarbonyl-protected amino acids (Bachem, Inc., Torrance, CA), purified by high performance liquid chromatography, and verified by mass spectrometry with an LCQ ion trap (Finnigan MAT, San Jose, CA). Mouse monoclonal antibodies against substituent peptides of human Edg-3 (amino acids 1–21), Edg-4 (amino acids 9–27), and Edg-5 (amino acids 303–322) with high interspecies homology were purified and used for Western blots at 0.1–0.3 μg/ml (26–28) (Antibody Solutions, Palo Alto, CA). A rabbit polyclonal antiserum to rodent and human Edg-2 was kindly provided by Dr. Jerold Chun (University of California San Diego). The cross-reactivity of each antibody with heterologous Edg proteins was less than 1%. Mouse monoclonal anti-human gelsolin antibody was purchased from Transduction Laboratories (Lexington, KY).

Cell Culture and Quantification of Cellular Protein Synthesis—Rat cardiac myocytes (RCMs) of 90% purity with up to 10% fibroblasts from 1-day-old Harlan Sprague-Dawley rat pups were cultured in Dulbecco's modified minimal Eagle's medium with penicillin and streptomycin ($DMEM^+$) and 10% fetal bovine serum at 37 °C in 5% CO_2 (29, 30). Replicate layers of RCM were preincubated with pertussis toxin for 6 h and C3 exoenzyme for 30 h. RCMs were serum-deprived 24 h before each study in $DMEM^+$ or $DMEM^+$ containing 1–100 μg/ml faf-BSA or 0.1–100 μg/ml of gelsolin, and the medium was replaced just before RCM stimulation at the beginning of a study. To quantify incorporation of [^{3}H]leucine into proteins by RCM, replicate monolayers of 3–4 × 10^3 RCM were serum-deprived and cultured in 12-well plates for 72 h with removal of medium and readdition of antagonists and stimuli in fresh medium every 24 h. In these and all other studies, LPA was added in an aliquot of 100 μg/ml faf-BSA equal to 1/50 the volume of medium in each well. This prevents LPA binding to non-cellular surfaces even at aqueous concentrations far below the critical micellar level of approximately 1 mM (5). One μCi of [^{3}H]leucine was added to wells, and incubation was continued for 12 h. RCM then were washed twice with 1 ml of phosphate-buffered saline, fixed in 0.5 ml/well of trichloroacetic acid at 5 g/100 ml of distilled water, held at room temperature for 15 min, and solubilized by incubation at 37 °C for 2 h in 0.2 ml of 5 g % sodium dodecyl sulfate for determination of radioactivity.

Western Blots—Replicate suspensions of 1 × 10^7 RCMs, fibroblasts from preparations of RCMs, and HTC4 cell transfectants were washed three times with 10 ml of ice-cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, resuspended in 0.3 ml of cold 10 mM Tris-HCl (pH 7.4) containing a protease inhibitor mixture (Sigma), 0.12 M sucrose, and 5% glycerol (v:v), and proteins were extracted (27). In initial analytical gels, aliquots of supernatant containing 1–100 μg of protein were electrophoresed, transferred, and immunostained (27).

Quantification and Analysis of Gelsolin and Serum Albumin Binding of LPA—Two polished lucite 8-cm diameter × 12-mm thickness disks with eight 10-mm diameter half-wells in each held a 10-kDa pore cut-off middle layer of acetylated dialysis membrane between the half-wells of each chamber. One half-well received 200 μl of either 5 μM faf-BSA or 2 μM gelsolin in phosphate-buffered saline with Ca^{2+} and Mg^{2+}, and the other received 200 μl of [^{3}H]LPA (150,000 dpm or 800,000 dpm in competition studies) in Ca^{2+}- and Mg^{2+}-containing phosphate-buffered saline with 20 μg/ml (0.3 μM) faf-BSA. Non-radioactive LPA at 0.1 nM–1 μM or a range of concentrations of binding inhibitors were added at equal concentrations to both half-wells of some sets. After 48 h of incubation with rolling at 4 °C, replicate aliquots of 50 μl were counted to determine the difference between dpm in a protein-containing half-well and in the non-protein half-well of each compartment. After normalization for recovery, which ranged from 49% to 70%, each difference was expressed as a percentage of dpm in the reference set lacking any inhibitor (100%). Plots of % of control binding *versus* LPA concentration were used to derive K_d values by the MacLigand program of Dr. Robert E. Williams (UCLA) and for corroboration by the LIGAND program of Biosoft (Cambridge, MA). Plots of % of control binding *versus* inhibitor concentration permitted calculation of IC_{50} values. Binding data were analyzed for statistical significance by one-way analysis of variance and Fisher's protected least significant difference (StatView, Abacus Concepts, Berkeley, CA).

Transfections and SRE Reporter Assay—Replicate monolayers of 0.3–1 × 10^6 RCMs in 12-well plates of $DMEM^+$ were lipotransfected with 100 ng/well of an SRE firefly luciferase reporter plasmid (16) and 5 ng/well of pRL-CMV *Renilla* luciferase vector (Promega) using FuGENE 6. After 24 to 30 h, the RCM were washed and covered with 1 ml of $DMEM^+$ before stimulation by LPA or a combination of monoclonal anti-Edg-4 antibody and phorbol myristate acetate (PMA). Each firefly luciferase luminometric value was corrected for process differences by division with the *Renilla* luciferase luminometric signal from the same well (31).

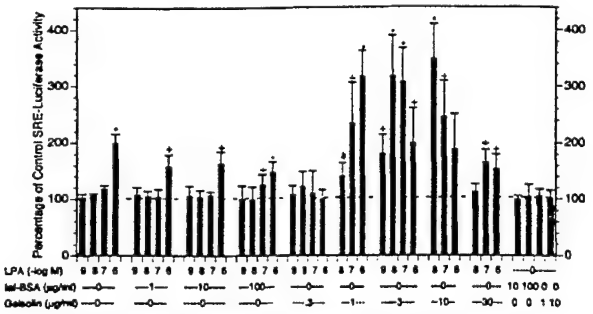

FIG. 1. **SRE luciferase reporter assay of nuclear signaling of rat cardiac myocytes by LPA.** Data and each *error bar* depict the mean ± S.D. of the results of three or four different studies. The medium alone control values were 2906, 3130, 8020, and 10,402 luminometer units. Human purified plasma gelsolin was used for the studies presented in this figure. The levels of significance of increases above medium controls were determined with a paired sample Student's *t* test. #, $p = 0.05$; +, $p < 0.05$; *, $p < 0.01$.

RESULTS

Direct nuclear signaling by LPA recruits the transcription proteins serum response factor and ternary complex factor to form a ternary complex with the SRE in promoters of numerous immediate-early growth-related genes. RCM transfected with an SRE luciferase reporter, permitting quantification of direct nuclear signaling, responded modestly only to 10^{-6} M LPA in protein-free medium, only to 10^{-6} M LPA in 1 and 10 μg/ml faf-BSA, and to 10^{-7} M and 10^{-6} M LPA in 100 μg/ml faf-BSA (Fig. 1). In contrast, delivery of LPA to RCM by 1, 3, and 10 μg/ml gelsolin resulted in significant responses at 10^{-9}–10^{-6} M LPA, with maximal mean increases in signal of 3- to 4-fold. The effectiveness of purified gelsolin was steeply dependent on concentration, with no responses at 0.3 μg/ml, maximum signals at 1–10 μg/ml, and lower than maximum responses at 30 μg/ml (Fig. 1). In two studies that included 100 μg/ml purified gelsolin, SRE luciferase signals were not significantly higher or lower than medium control values at 10^{-8}–10^{-6} M LPA. In limited studies, 10^{-8} M and 10^{-7} M LPA delivered by 3 μg/ml human recombinant gelsolin elicited respective mean increases (±S.D.) in SRE luciferase signals to 312 ± 48% and 274 ± 60% of control (both $p < 0.01$). The presentation of LPA to RCMs by gelsolin thus attained a peak at concentrations lower than 10% that in plasma and decreased at levels 20% or more of that in plasma. In contrast, gelsolin did not enhance S1P-evoked SRE luciferase responses in parallel experiments (data not shown). Furthermore, neither purified or recombinant gelsolin nor faf-BSA alone evoked SRE luciferase responses (Fig. 1).

As RCM do not proliferate in culture, when assessed by increases in cell counts, the functional consequences of their activation by LPA were investigated by measuring enhancement of protein synthesis characteristic of myocyte hypertrophy, as quantified by uptake of [^{3}H]leucine into trichloroacetic acid-precipitated cellular proteins. The level of protein synthesis by RCM was increased by LPA delivered by purified plasma gelsolin (Fig. 2). The increases in incorporation of [^{3}H]leucine were significant at 10^{-8}–10^{-6} M LPA with 3 μg/ml and 10 μg/ml gelsolin and attained a peak at 10^{-7} M and 10^{-6} M with 3 μg/ml and 10^{-8} M and 10^{-7} M with 10 μg/ml gelsolin. In limited studies, 10^{-8} M and 10^{-7} M LPA delivered by 3 μg/ml human recombinant gelsolin elicited respective mean increases (±S.D.) in incorporation of [^{3}H]leucine to 194 ± 44% and 236 ± 18% ($p < 0.05$ and <0.01, respectively) of control. There was a marginally significant increase in RCM protein synthesis with

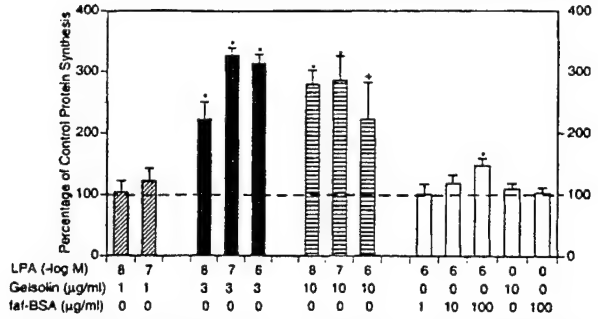

FIG. 2. **Stimulation by LPA of increases in incorporation of [^{3}H]leucine into proteins by rat cardiac myocytes: purified gelsolin and LPA concentration dependence relationships.** Data and each *error bar* depict the mean ± S.D. of the results of three different studies. The medium alone control values (100%) were 24,902, 18,655, and 29,342 dpm. The levels of significance of changes from control levels were determined with a paired sample Student's *t* test. #, $p = 0.05$; +, $p < 0.05$; *, $p < 0.01$.

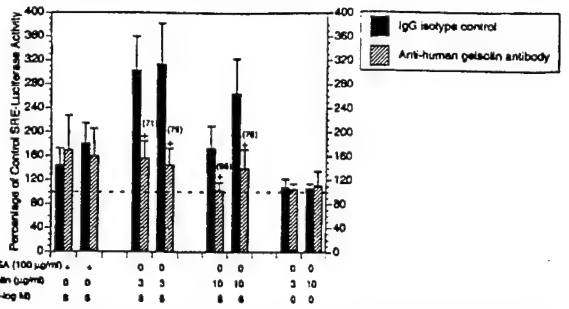

FIG. 3. **Inhibition by monoclonal anti-gelsolin antibody of delivery of LPA to rat cardiac myocytes by human purified gelsolin.** Data and each *error bar* depict the mean ± S.D. of the results of three different studies. The concentration of anti-gelsolin antibody and isotype control IgG was 10 μg/ml. The levels of significance of inhibition of SRE luciferase responses were determined with a paired sample Student's *t* test. #, $p = 0.05$; +, $p < 0.05$; *, $p < 0.01$. The *number* in *parentheses* above each *bar* showing significant suppression of responses is the mean percentage suppression of the corresponding response with control IgG.

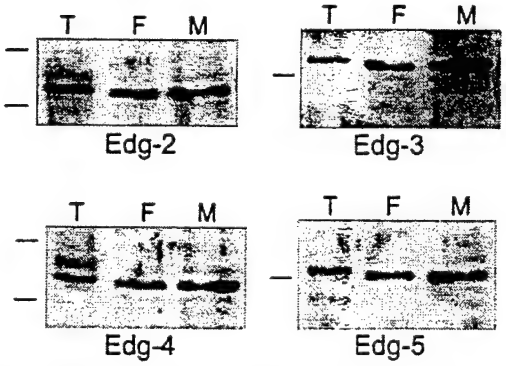

FIG. 4. **Western blot analyses of the expression of Edg-2, -3, -4, and -5 receptors by rat cardiac myocytes and fibroblasts.** The three samples are 2 μg of protein from HTC4 rat hepatoma cells (*T*), which were stably transfected with the respective EdgRs, 10 μg of protein from rat cardiac fibroblasts (*F*), which constitute up to 10% of cells in cardiac myocyte preparations, and 4 μg of protein from rat cardiac myocytes of 90% purity (*M*). No Edg-3, -4, or -5 receptor antigen was detected in untransfected HTC4 cells with monoclonal antibodies, but a low level of Edg-2R antigen was revealed by the rabbit anti-mouse Edg-2R antibodies. The marginal lines show the positions of the 45-kDa and 66-kDa protein molecular mass standards for Edg-2 and -4 and the 45 kDa molecular mass standard for Edg-3 and -5.

10^{-6} M LPA delivered by 100 μg/ml faf-BSA. In contrast, LPA delivered by 1 μg/ml gelsolin or by 1 μg/ml or 10 μg/ml faf-BSA had no effect on RCM protein synthesis (Fig. 2). The maximal responses to gelsolin-LPA far exceeded in magnitude those evoked by faf-BSA-LPA. Neither gelsolin nor faf-BSA alone altered RCM protein synthesis.

The identity of gelsolin as the protein delivering LPA to RCM was confirmed by the suppression of SRE luciferase responses with a neutralizing mouse monoclonal antibody to human gelsolin. RCM SRE luciferase responses similar in magnitude to those described in Fig. 1 were again evoked when LPA was delivered by gelsolin or faf-BSA (Fig. 3). In contrast, neither gelsolin alone or gelsolin with the monoclonal anti-gelsolin antibody increased SRE luciferase reports. The responses elicited by gelsolin-LPA, but not faf-BSA-LPA, were suppressed significantly by means of more than 70% with mouse anti-gelsolin antibody (Fig. 3).

Monoclonal antibodies to the EdgRs that bind and transduce signals from LPA recently were shown to elicit growth-related responses of some cells when introduced with PMA (32). Thus the EdgRs of RCM were analyzed immunochemically first and then for their capacity to transduce antibody-induced signals to determine if gelsolin influenced any receptor or post-receptor elements of RCM responses in addition to LPA delivery. RCM expression of Edg-2 and -4 receptors for LPA and Edg-3 and -5 receptors for S1P was examined in Western blots developed with the monoclonal antibodies to human and rodent Edg-3, -4, and -5 receptors and rabbit IgG polyclonal antibodies to human and rodent Edg-2Rs with stimulatory potential (Fig. 4). One predominant protein antigen was observed with each anti-EdgR antibody in extracts of RCM that was the same size as the protein detected by that antibody in extracts of HTC4 rat hepatoma cell transfectants bearing the corresponding recombinant EdgR (21, 31). The apparent quantity of each EdgR antigen extracted from purified cardiac fibroblasts, which represent up to 10% of the cells in RCM preparations, was lower than that in RCMs despite the application of 2.5 times more protein from the fibroblasts (Fig. 4). RCMs thus appear to be the principal cells in fresh preparations that express EdgRs and can respond by EdgR-dependent mechanisms both to LPA and anti-EdgR antibodies combined with PMA. Two toxins active against transductional components required for EdgR signaling were employed to determine if RCM EdgRs signal by the expected mechanisms. Both pertussis toxin and C3 exoenzyme separately significantly suppressed the increases in SRE luciferase activity induced by LPA delivered to RCM by either 100 μg/ml faf-BSA or 3 μg/ml gelsolin (Table I). Application of pertussis toxin and C3 exoenzyme together inhibited SRE luciferase reporter responses to 10^{-7} M LPA with 3 μg/ml gelsolin by a mean of 83% ($n = 2$).

As Edg-4R was one of the most prominent LPA receptors of RCMs and monoclonal anti-Edg-4R antibody with PMA has evoked SRE luciferase responses in other cells (32), concentrations of anti-Edg-4R antibody and PMA, which alone did not elicit signals, were applied to RCM in several combinations (Table II). These combinations of monoclonal anti-Edg-4R antibody and PMA evoked mean SRE luciferase responses of RCM from 194% to 235%, which were similar in magnitude to those induced by LPA with gelsolin. However, gelsolin did not significantly modify the signals attained by anti-Edg-4R antibody-based stimulation (Table II). Thus gelsolin does not appear to have major effects on post-EdgR cellular elements of signaling of RCMs.

The capacity of concentrations of gelsolin lower than 10% that of normal plasma to deliver LPA to RCMs with optimal efficiency suggested high affinity binding of LPA to gelsolin.

TABLE I

Inhibition by selective toxins of SRE-luciferase responses to LPA

Protein concentrations were 100 μg/ml faf-BSA and 3 μg/ml gelsolin. Each percentage inhibition value is the mean ± range for results of two studies conducted in duplicate. $p < 0.01$ by a paired sample Student's *t* test.

	faf-BSA	Purified gelsolin
10^{-7} M LPA + pertussis toxin	62 ± 3	61 ± 8
10^{-7} M LPA + C3 exoenzyme	70 ± 11	58 ± 7

TABLE II

Lack of effect of gelsolin on anti-Edg-4 receptor monoclonal antibody stimulation of nuclear responses in rat cardiac myocytes

Each value is the mean ± S.D. of three separate studies. In the absence of gelsolin, anti-Edg-4 monoclonal antibody (MoAb) plus PMA at respective concentrations of 0.1/0.3, 0.1/1.0, 1.0/0.3, and 1.0 μg/ml/1.0 ng/ml increased SRE-luciferase responses significantly to corresponding levels of 194 + 45%, 235 + 55%, 216 + 63%, and 228 + 56% (mean ± S.D.) of medium controls, which were set at 100% for quantification of the effects of gelsolin. The same concentrations of a similar anti-Edg-3 monoclonal antibody plus PMA did not stimulate SRE luciferase responses despite expression of Edg-3Rs (Fig. 4).

[Anti-Edg-4 MoAb]	[PMA]	Purified gelsolin (μg/ml)	
		3	10
μg/ml	*ng/ml*	*% gelsolin-free control response*	
0.1	0.3	125 ± 24	108 ± 5
0.1	1.0	106 ± 16	105 ± 28
1.0	0.3	101 ± 3	106 ± 18
1.0	1.0	113 ± 20	101 ± 6

The results of analyses of equilibrium binding of [^{3}H]LPA to gelsolin and faf-BSA showed greater displacement from the former by all concentrations of non-radioactive LPA but especially large differences between gelsolin and faf-BSA at 3×10^{-8} M to 3×10^{-7} M (Fig. 5). Both recombinant and purified human gelsolin demonstrated similarly greater displacement of [^{3}H]LPA at low LPA concentrations. Purified and recombinant gelsolin had respective mean K_d values ± S.E. ($n = 3$) of 6.2 ± 2.5 nM and 32 ± 7.8 nM, as contrasted with 357 ± 64 nM for faf-BSA. Assessment of valences of the binding proteins indicated a range of 1.2 to 1.8 for gelsolin and 3.2 to 5.6 for faf-BSA. S1P and lysophosphatidylcholine at 10^{-6} M both failed to displace [^{3}H]LPA from gelsolin.

A relationship between gelsolin binding of LPA and of PIP2 was examined by PIP2 competitive inhibition of both gelsolin binding of [^{3}H]LPA and cellular presentation of LPA by gelsolin. A 300-fold molar excess of PIP2 inhibited LPA binding to gelsolin by a mean of 31% (Fig. 6*A*), and a 100-fold molar excess of PIP2 inhibited LPA presentation to RCM by a mean of 56% for nuclear signaling (Fig. 6*B*) and 38% for protein synthesis (Fig. 6*C*). In contrast, there was no significant inhibition of LPA binding to faf-BSA or of faf-BSA enhancement of LPA effects on cardiomyocytes by PIP2 (data not shown). Substituent peptides of gelsolin (gel P1 and gel P2), each of which represents one of the two PIP2-binding sites of intact gelsolin and of another actin-binding protein termed villin (villin P), were used as gelsolin competitive inhibitors at concentrations 3–300-fold higher than 0.03–0.3 μM intact gelsolin (13, 14, 25). Gel P1 and gel P2 significantly suppressed binding of [^{3}H]LPA to 0.3 μM intact gelsolin at 3-fold and 30-fold higher molar concentrations (Fig. 6*A*). Gel P1 and gel P2 significantly suppressed signaling of SRE luciferase by LPA-0.03 μM gelsolin at 30-fold and 300-fold higher molar concentrations (Fig. 6*B*). The two gelsolin peptide sites of PIP2 binding also significantly inhibited induction of RCM protein synthesis by LPA with 0.03 μM gelsolin at a 300-fold higher molar concentration (Fig. 6*C*). In contrast, a control nonsense sequence gelsolin peptide 2 had no effect on intact gelsolin binding or delivery of LPA (Fig. 6).

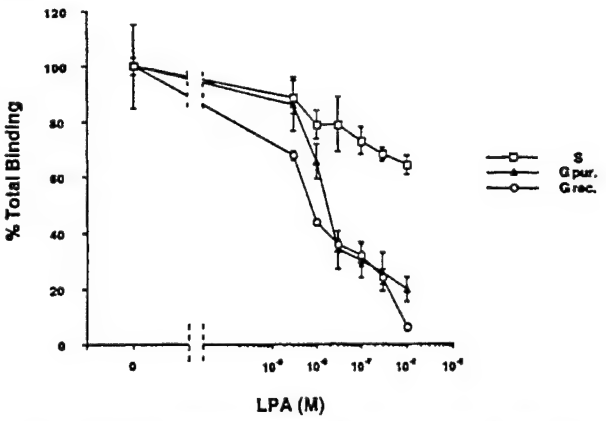

FIG. 5. **LPA concentration dependence of displacement of [^{3}H]LPA bound to purified human plasma gelsolin (*G pur.*), human recombinant gelsolin (*G rec.*) and fatty acid-free bovine serum albumin (*S*).** Each point is the mean ± S.E. of the results of two different studies carried out in duplicate. The mean net specific binding of 150,000 dpm of [^{3}H]LPA to 5 μM faf-BSA was 52,822 and 56,582 dpm, to 2 μM plasma gelsolin was 9,740 and 10,851 dpm, and to 2 μM recombinant gelsolin was 20,412 and 20,390 dpm in the two studies in the absence of non-radioactive LPA (100%).

Villin P, which is structurally homologous to gelsolin PIP2 binding peptides, also inhibited gelsolin binding and cellular presentation of LPA at similar molar concentration ratios with intact gelsolin. None of the gelsolin inhibitory peptides significantly altered faf-BSA binding or delivery of LPA.

DISCUSSION

The expression of one or more EdgRs by all but a few types of mammalian cells has suggested that the activities of LPA and S1P are regulated principally by alterations in their effective local concentrations. The relative roles of production, non-receptor protein binding, and biodegradation in the determination of concentrations of LPA at cell-surface EdgRs have not been elucidated in physiological settings. The amount of LPA generated is a function of membrane perturbation of cellular sources and recruitment of a sequence of phospholipases, which are induced by diverse stimuli (1–3). Many lysophospholipases with different cellular localization, substrate specificity, and susceptibility to stimulation and inhibition may degrade or otherwise inactivate LPA (33). Thus, LPA is produced and biodegraded at sites of tissue reactions in the course of many physiological responses and pathological processes. Less is known of the mechanisms for stabilization of LPA in tissues and specific cellular delivery of LPA.

Plasma gelsolin, which is derived principally from myocytes (14, 34), binds LPA with an affinity similar to that of EdgRs and, exceeding, that of serum albumin or other known plasma proteins (Fig. 5). The identity of gelsolin as the LPA-binding protein of purified plasma preparations was verified by the similar activity of recombinant gelsolin (Fig. 5) and the neutralizing effect of monoclonal anti-gelsolin antibody (Fig. 3). This implies that LPA derived from any primary cellular source first will bind with low affinity to the much greater density of serum albumin sites and then may be captured effectively by high affinity binding to gelsolin. The present data suggest that gelsolin could serve two functions in regulating expression of LPA activity. Gelsolin concentrations in plasma normally are 150 to 250 μg/ml and rarely decrease to lower than 60 μg/ml even in cases of extensive trauma, where there is extreme dilution and actin-trapping (35–39). At such high plasma concentrations, therefore, the first function of gelsolin may be as a high affinity plasma carrier, which protects a portion of circu-

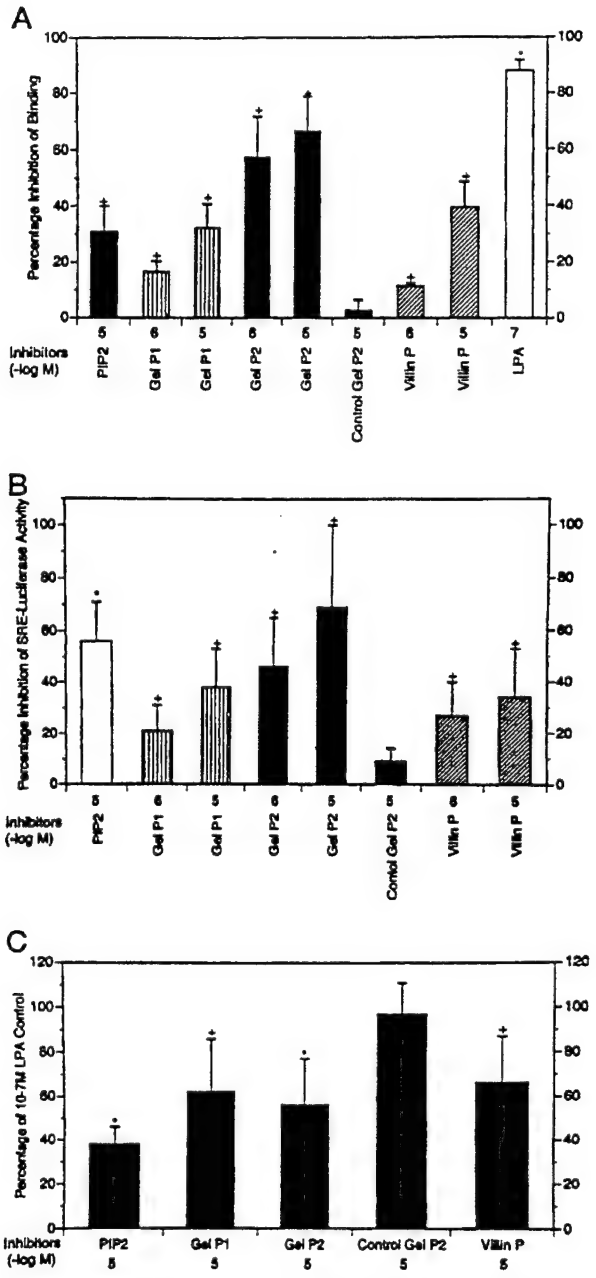

FIG. 6. **Competitive inhibition by PIP2 and binding site peptides of actin-binding proteins of [^{3}H]LPA binding to human purified gelsolin (*A*) and of LPA-gelsolin stimulation of SRE luciferase (*B*) and incorporation of [^{3}H]leucine into proteins (*C*) in rat cardiac myocytes.** *A*, inhibition of binding of 800,000 dpm of [^{3}H]LPA to 0.3 μM human purified plasma gelsolin. Data and each *error bar* depict the mean ± S.E. of the results of three or four different studies, except for gelsolin peptide 1 and control gelsolin peptide 2, which are the mean of duplicate results. The control levels of binding of [^{3}H]LPA alone (0% inhibition) ranged from 6,802 to 20,425 dpm. *B*, inhibition of stimulation of SRE luciferase activity by 10^{-7} M LPA, 3 μg/ml gelsolin. Data and each *error bar* depict the mean ± S.D. of the results of three different studies. The control levels of SRE luciferase with LPA and gelsolin alone (0% inhibition) were 9,332, 3,241, and 17,089 luminometer units. *C*, inhibition of stimulation of incorporation of [^{3}H]leucine into proteins by LPA and gelsolin. Data and each *error bar* depict the mean ± S.D. of the results of three different studies. The 3 μg/ml gelsolin and 10^{-7} M LPA alone control values (0% inhibition) were 93,629, 84,710, and 81,505 dpm. The levels of significance of mean percentage inhibition in A, B, and C were determined with a paired sample Student's *t* test. #, $p = 0.05$; +, $p < 0.05$; *, $p < 0.01$.

lating LPA from biodegradation, prevents LPA from binding to numerous possible low affinity proteins, and suppresses LPA activation of endothelial cells, platelets, and circulating leukocytes (Figs. 1 and 2). At the lower concentrations of gelsolin observed in fluids of injured or inflamed tissues, which range from 1% to 10% of that in plasma,[2] its second function may be delivery of LPA to myocytes and some other types of cells with a greater effectiveness than that characteristic of serum albumin (Figs. 1 and 2). Confirming investigations by Stossel and co-workers[2] have characterized delivery of LPA to Swiss 3T3 cells by recombinant human gelsolin added to plasma from mice genetically totally deficient in gelsolin. LPA stimulation of the formation of actin stress fibers in 3T3 cells was enhanced maximally by gelsolin at approximately 1% normal plasma levels and was suppressed by normal plasma concentrations. The delivery of LPA to specific EdgRs by low concentrations of gelsolin may be driven by gradients of gelsolin concentration, which would be higher at the surface of cells producing gelsolin, as well as gradients of LPA affinity. That delivery of LPA to cellular receptors is the principal function of gelsolin in this context was shown by its lack of effect on immunospecific stimulation. RCMs express functional EdgRs for both LPA and S1P (Fig. 4; Table I). The nuclear signaling to SRE by a combination of phorbol ester and monoclonal anti-Edg-4 antibody at separately inactive concentrations was unaffected by gelsolin, which is consistent with the absence of any direct receptor or post-receptor effect of gelsolin (Table II).

Maximum stimulation of nuclear responses and enhancement of protein synthesis in RCM were attained when LPA was delivered by gelsolin at concentrations of 10% or lower than that in normal plasma (Figs. 1 and 2). Leakage of cytoplasmic gelsolin from ischemic or otherwise injured RCM and exudation of plasma gelsolin from permeabilized vasculature into inflamed myocardium at low concentrations thus initially would enhance locally the capacity of LPA to suppress cardiomyocyte apoptosis (40) and to evoke restorative events. Under different myocardial conditions, low levels of gelsolin may enhance LPA induction of greater contractility and/or promote LPA stimulation of myocardial hypertrophy. Of note, the fold increase in protein synthesis evoked by 100 nM LPA with 3 μg/ml gelsolin is greater than that induced by 1 μM norepinephrine or 1% fetal calf serum in other studies (29). However, at gelsolin concentrations above 20% those in normal plasma, later in the evolution of tissue responses plasma gelsolin may trap LPA, prevent effective delivery to EdgRs, and thereby diminish many of the biological effects of LPA. Thus gelsolin has the capacity to enhance and suppress the effects of LPA on myocytes and possibly other types of cells.

Molar excesses of PIP2 and substituent peptides of gelsolin constituting the PIP2-binding sites blocked LPA binding to gelsolin by 50% or more and inhibited LPA stimulation of both nuclear responses and protein synthesis (Fig. 6). This is consistent with a previous report of LPA regulation of the actin-binding, and -cleaving activity of gelsolin, which was similar to the effect of PIP2 and presumed to be attributable to LPA interactions with PIP2 sites (12). Gelsolin did not bind S1P nor deliver S1P to RCMs. Other plasma and non-receptor cellular proteins may bind S1P with effects similar to those of gelsolin for LPA but have not yet been identified. The mechanisms by which gelsolin delivers LPA to cells effectively remain to be elucidated but may include a gelsolin-selective docking site as well as bivalent and, thus, higher net affinity presentation. Also remaining for future studies are questions of any protec-

[2] M. May, C. Lilly, K. Haley, M. Sunday, C. Sheils, P. Allen, T. P. Stossel, and J. M. Drazen, personal communication.

tive or carrier functions of gelsolin for LPA and any ability of gelsolin to facilitate transport of LPA into cells or across tissue boundaries.

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Lysophospholipid Growth Factors

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SUMMARY

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are the biologically predominant members of a family of mediators termed lysophospholipid growth factors (PLGFs) or lysophospholipids (LPLs). These amphipathic phospholipids are generated by platelets, macrophages, other leukocytes, epithelial cells, and some tumors in amounts that result in micromolar concentrations in serum and some tissue fluids. A family of G protein-coupled receptors bind LPA and S1P to transduce signals stimulating cellular proliferation, differentiation, survival, and cellular functions. LPA and S1P have roles in hemostasis, cardiovascular regulation, wound healing, cytoprotection, immunity, inflammation, and organ system development normally, and in the pathogenesis of some types of *cancer*.

BACKGROUND

Discovery

Lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P), and other LPLs/PLGFs are amphipathic metabolic products of membrane phospholipids in stimulated cells, which are present in many mammalian physiological fluids at up to micromolar concentrations and have diverse effects on cellular proliferation, differentiation, survival, and functions (Moolenaar, 1995; Spiegel and Milstien, 1995; Tokumura, 1995; Spiegel and Merrill, 1996; Heringdorf *et al.*, 1997; Moolenaar *et al.*, 1997; An *et al.*, 1998a; Goetzl and An, 1998; Lee *et al.*, 1999b; Tigyi *et al.*, 1999; Goetzl and Lynch, 2000). The PLGFs/LPLs were discovered first chemically in the mid- to late 1800s among other organic solvent-extractable components of cells and tissues. The discovery of LPA as a biologically active acidic phospholipid dates back to the late 1940s and 1950s (Vogt, 1949, 1957; Gabr, 1956) when it was isolated and identified as a stimulator of intestinal smooth muscle contraction. The only unifying structural motif of LPLs/PLGFs is the ubiquitous presence of a phosphate ester moiety and a hydrocarbon chain, which are linked to glycerol or serine backbone.

Glycero-PLGFs/LPLs, exemplified by LPA, are members of the phospholipid family which predominates quantitatively among lipid structural constituents of cellular membranes. Sphingolipids were named for their shared sphingoid backbone and represent a quantitatively diminutive family compared with phospholipids. However, as shown by the protean biological activities of S1P, sphingolipids are one of the structurally and functionally most complex classes of biological mediators. As for our evolving understanding of other bioactive lipids, the appreciation of crucial roles of PLGFs/LPLs in cellular biology derives from recent observations of their rapid generation from cell membrane precursors, ability to directly regulate lipid-metabolizing enzymes (Baker and Chang, 2000), often transient appearance in relation to cellular responses, capacity to move with proteins in the planes of membranes, and potent effects on critical activities of cells both as intracellular messengers and extracellular mediators.

Alternative names

Lysoglycerophospholipids, lysosphingophospholipids, phosphatidates.

Structure

The LPLs all are complex lipids with one phosphate, one free hydroxyl group, and a medium- or long-chain fatty acid on a glycerol or sphingoid backbone. The glycerolipid PLGFs/LPLs usually contain one phosphate ester moiety and an ester- or ether-linked hydrocarbon chain. Although many of them contain a hydroxyl group in the sn-1 or sn-2 position of the glycerol backbone, its presence is not essential for biological activity, as naturally occurring 1,2-cyclic-phosphatidic acid (Fischer *et al.*, 1998; Liliom *et al.*, 1998; Kobayashi *et al.*, 1999), as well as short-chain (Jalink *et al.*, 1994) and/or long-chain phosphatidic acids (Siddiqui and English, 1996, 1997) elicit similar cellular responses. The sphingolipid phosphates implicated as extracellular mediators include S1P (Postma *et al.*, 1996), sphingosylphosphorylcholine, psychosine, glucopsychosine (Himmel *et al.*, 1998), and ceramide-1-phosphate (Gomez-Munoz *et al.*, 1995).

Main activities and pathophysiological roles

The PLGFs/LPLs serve as both extracellular mediators and intracellular messengers for many types of cells (see the sections on *In vitro* activities and *In vivo* biological activities). In addition to their primary effects, PLGFs/LPLs have prominent secondary effects on cells through their abilities to alter production of protein growth factors, act synergistically with other growth factors, and modify expression of receptors for protein growth factors.

The current intense focus on PLGFs/LPLs was initiated in part by the discovery that many of their effects as extracellular mediators for diverse types of cells are transduced by a novel family of highly specific G protein-coupled receptors (GPCRs) (see chapter on Lysophospholipid growth factor receptors). These GPCRs were identified first as elements of expression of immediate-early responses of endothelial cells to differentiating stimuli, and thus have been designated tentatively the endothelial differentiation gene receptors (Edg Rs). The consideration of PLGFs/LPLs in this section and of Edg Rs in the companion section will be directed principally to descriptions of their distinctive cytokine-like effects in cellular differentiation, survival, proliferation, and cytoskeleton-based functions, and of new findings about their possible roles in human diseases.

GENE AND GENE REGULATION

As PLGFs/LPLs are generated and biodegraded by complex cellular pathways involving multiple enzymes (**Table 1**), expression of the genes encoding pathway-controlling enzymes and their regulatory factors determines the net levels of each LPL during any tissue reaction. The predominant pathways and enzymes also may differ in each type of cell, which results in a level of complexity precluding at present a meaningful tabulation of genes controlling the family of PLGFs/LPLs.

Table 1 Rate-controlling and regulatory enzymes of lysolipid phosphate biosynthesis

Activity	LPA	S1P
Release and metabolism of membrane precursor (product)	Phospholipase D and/or phospholipase C + DAGK with sphingomyelinase (phosphatidic acid)	Sphingomyelinases (ceramide) Ceramidase (sphingosine)
Conversion to LPL (product)	Secretory PLA_2 (LPA)	Sphingosine kinase (S1P)
Biodegradation of LPL	Phosphatidate PH	PP-lyase
	Lysophospholipases	
	LPA acyltransferase	

DAGK, diacylglycerol kinase; PH, phosphohydrolase; PP, pyridoxal phosphate-dependent.

Accession numbers

See Protein accession numbers.

Chromosome location

The location of the gene for each lipid-metabolizing enzyme is available from standard databases.

Cells and tissues that express the gene

These studies are not complete, but available data suggest expression of genes encoding the LPL synthetic systems principally in megakaryocytes (platelets), macrophages, some other leukocytes, epithelial cells, and some tumor cells.

PROTEIN

Accession numbers

Critical lipid-metabolizing enzymes of the systems for generating LPA and S1P are as follows:
Human:
Acid sphingomyelinase: M59916
Neutral sphingomyelinase: AJ222801
Ceramidase: U70063
Phospholipase C (PLC): α D16234, γ M34667
Phospholipase D (PLD): L11701
Diacylglycerol kinase: X62535
Secretory PLA_2: AF112982
Lysophosphatidic acid acyltransferase: AF000237
Mouse:
Sphingosine kinase: AF068749

Sequence

These are available through accession numbers.

Description of protein

The enzymes responsible for production of LPA and S1P are phospholipases (PLs), sphingolipases, and highly specialized lipid kinases. After sphingomyelinase conditioning of plasma membrane vesicles released from activated platelets, leukocytes, epithelial cells, and some tumors, PLC- and/or PLD-dependent mechanisms liberate phosphatidic acid, which is converted to LPA by secretory PLA_2 and possibly other PLs (Table 1) (Gaits *et al.*, 1997). Analogously, S1P is generated by the sequential actions of sphingomyelinases, ceramidase, and sphingosine kinase (Table 1) (Hannun, 1994; Spiegel and Merrill, 1996; Schissel *et al.*, 1998; Tomiuk *et al.*, 1998). PLA_2 and sphingosine kinase are the dominant rate-controlling enzymes in the respective synthetic pathways (Fourcade *et al.*, 1995; Higgs *et al.*, 1998; Kohama *et al.*, 1998). Concurrent degradative activities of a series of lysophospholipases, lysolipid phosphatases, acyltransferases, and an S1P-specific lyase contribute significantly to the courses of appearance and net maximal concentrations of LPA and S1P attained in any reaction (Waggoner *et al.*, 1996; Eberhardt *et al.*, 1997; Kai *et al.*, 1997; Wang *et al.*, 1997; Brindley and Waggoner, 1998; Mandala *et al.*, 1998; Roberts *et al.*, 1998). The dependence of tissue and fluid concentrations of LPA and S1P on multiple LPL-generating and -metabolizing enzymes suggests that a genetic defect in any one will alter the respective downstream pathways with functional significance.

CELLULAR SOURCES AND TISSUE EXPRESSION

Cellular sources that produce

LPA was definitively characterized structurally and as a serum and incubated-plasma vasoactive and platelet-active factor in the late 1970s (Tokumura *et al.*, 1978; Schumacher *et al.*, 1979). Three stimulus-coupled cellular synthetic schemes have been implicated in LPA generation and, by analogy S1P production. (1) Generation by degradation of complex membrane phospholipids. After sphingomyelinase conditioning of plasma membrane vesicles released from activated cells, PLC- and/or PLD-dependent mechanisms liberate phosphatidic acid, which is converted to LPA by secretory PLA_2 and possibly other PLs (Table 1). (2) Generation by lipid kinases. In thrombin-activated platelets diacylglycerol kinase is thought to generate PA, which in turn is cleaved to LPA by secretory PLA_2 (Lapetina *et al.*, 1981a,b). (3) Generation by oxidative degradation. Siess and colleagues have shown the production of LPA in minimally oxidized low-density lipoprotein, by mechanisms similar to those that generate PAF-derivatives (Siess *et al.*, 1999). The serum concentration of LPA is micromolar, in contrast with nanomolar levels in fresh plasma, suggesting that platelets are a major source of LPA (Gerrard and

Robinson, 1984; Eichholtz *et al.*, 1993). Macrophages, some other types of leukocytes, many epithelial cells, ovarian cancer cells, and some other tumor cells also produce and retain LPA at rates which result in intracellular concentrations ranging up to 30–60 μM (Tokumura *et al.*, 1999). The interactions of LPA with various intracellular lipid-binding proteins have not been studied to date.

Extracellular LPA is bound by serum albumin with an apparent K_d of 350 nM, which enhances cellular delivery and effective potency (Tigyi and Miledi, 1992; Thumser *et al.*, 1994; Goetzl *et al.*, 1999c). Recent analyses have shown high-affinity binding of LPA to plasma gelsolin, with an apparent K_d of 6–7 nM, which delivers LPA to some types of cells more efficiently and with more potent activity than serum albumin (Goetzl *et al.*, 1999c). LPA binds to the two phosphatidylinositol diphosphate (PIP_2) sites of gelsolin in competition with PIP_2. Cellular delivery of LPA by gelsolin is most effective at concentrations of 1% to up to 10% of those in normal plasma. At concentrations < 10% of those in normal plasma, gelsolin traps LPA and prevents access to cells with an efficiency which may explain the lack of effect of LPA in plasma on endothelial cells normally. The fact that lower ratios of gelsolin to LPA may lead to decreased binding affinity has suggested negative site cooperativity. Thus plasma gelsolin may carry LPA in an inactive state and then deliver it to cells in an active state when gelsolin levels drop in plasma and tissue fluids, as a result of dilution and binding to actin released from injured cells. This possibility is supported by findings of gelsolin concentrations optimal for cellular delivery of LPA in fluids of burned tissues and airway secretions of inflammatory lung diseases (Goetzl *et al.*, 1999c).

Sphingolysolipid phosphate mediators, such as S1P, are formed during turnover and degradation of membrane sphingolipids by diverse sphingomyelinases and downstream enzymes in numerous types of cells (Spiegel and Milstien, 1995; Spiegel and Merrill, 1996) (Table 1). Plasma and serum concentrations of S1P are in the high nanomolar to micromolar range with extensive binding normally to serum albumin and possibly other proteins, but not gelsolin.

Eliciting and inhibitory stimuli, including exogenous and endogenous modulators

Platelet adherence and aggregation, induced by a wide range of platelet-activating factors, and activation of macrophages and fibroblasts by relevant cytokines stimulate generation of LPA and S1P. PDGF stimulation of fibroblasts and α_1-adrenergic stimulation of adipocytes lead to the secretion of LPA into the extracellular medium. Thrombin stimulation of platelets causes the release of stored S1P into the bloodstream (Yatomi *et al.*, 1995).

RECEPTOR UTILIZATION

Two distinct types of GPCRs bind LPLs specifically and consequently transduce diverse cellular signals by associating with one or more G proteins. The Edg R family is composed of two subfamilies in which members are linked by higher levels of structural homology and specific recognition of the same LPL ligand (see chapter on Lysophospholipid growth factor receptors). Edg-1, -3 and -5 exhibit approximately 50% amino acid sequence identity and bind S1P, but not LPA, with high-affinity (An *et al.*, 1997b, 1998a; Zondag *et al.*, 1998). Edg-2, -4, and -7 represent a second cluster of structural similarity and bind LPA, but not S1P, with high affinity (Hecht *et al.*, 1996; An *et al.*, 1997a, 1998b; Bandoh *et al.*, 1999). Each Edg R also shows a distinctive profile of association with G proteins, which explains in part the differences in cellular activities between members of a subfamily. The LPL ligands for Edg-6 and Edg-8 have not been defined to date. *Xenopus* oocytes and some rodent cells express a second type of GPCR for LPA, termed PSP24, which is modestly homologous with the GPCR for phospholipid platelet-activating factor but not with Edg Rs (Guo *et al.*, 1996; Kawasawa, *et al.*, 1998). PSP24 transduces LPA-evoked oscillatory Cl^-currents through activation of the inositol trisphosphate-Ca^{2+} system and is most highly expressed in the central nervous system of mice, but its physiological roles are still under investigation.

IN VITRO ACTIVITIES

In vitro findings

The capacity of S1P to act as a potent intracellular messenger was suggested initially by its compartmentalized generation and concentration in cells responding to protein growth factors (Rani *et al.*, 1997; Melendez *et al.*, 1998). The facts that inhibitors of the sphingosine kinase, which controls synthesis of S1P, suppressed transduction of signals from receptors for some protein growth factors selectively and that exogenous S1P reversed this suppression support a role for S1P as an intracellular messenger (Spiegel and

Merrill, 1996). LPA has been implicated as an intracellular mediator of synaptic vesicle formation (Schmidt *et al.*, 1999).

The principal biological activities of LPA and S1P are as extracellular mediators, which have three basic types of effects (**Table 2**). The first are growth-related and include proliferation, differentiation, enhanced survival, and decreased sensitivity to apoptosis of diverse types of cells (Van Corven *et al.*, 1989, 1993; Tigyi *et al.*, 1994; Tokumura *et al.*, 1994; Piazza *et al.*, 1995; Wu *et al.*, 1995; Cuvillier *et al.*, 1996; Seufferlein *et al.*, 1996; Inoue *et al.*, 1997; Levine *et al.*, 1997; Herrlich *et al.*, 1998; Holtsberg *et al.*, 1998; Koh *et al.*, 1998; Dixon *et al.*, 1999; Goetzl *et al.*, 1999a; Hisano *et al.*, 1999; Pebay *et al.*, 1999; Pyne *et al.*, 1999; Sato *et al.*, 1999). The second are cytoskeleton-based functional effects, which include shape change, altered adherence, chemotaxis, contraction, and secretion (Tigyi and Miledi, 1992; Imamura *et al.*, 1993; Kolodney and Elson, 1993; Bornfeldt *et al.*,

Table 2 Biological activities of LPA and S1P

Activities	LPA	S1P
Growth-related effects		
Stimulate cellular proliferation	Fibroblasts	Fibroblasts
	Renal tubular cells	Monocytes
	Mesangial cells	T cells
	Smooth muscle cells (vascular)	Cancer cells
	Keratinocytes	
	T cells	
	Cancer cells	
Increase cellular survival	Macrophages	
	B lymphocytes	
Suppress apoptosis	Renal tubular cells	Fibroblasts
	Cardiac monocytes	Endothelial cells
	T cells	T cells
	Monocytes	Monocytes
		Oocytes
Cytoskeleton-based responses		
Cell morphology	Neurite retraction	Neurite retraction
Actin cytoskeletal remodeling	Myocyte hypertrophy	Myocyte hypertrophy
Cell–cell/cell–matrix adhesion	Focal adhesion	Fibronectin matrix assembly
	Platelet aggregation	Platelet aggregation
	Leukocyte–endothelial interactions	Leukocyte–endothelial interactions
Chemotaxis/kinesis	Tumor cells (transcellular)	Neutrophils (inhibition)
	Endothelial cells	Endothelial cells
Secretion	Neurotransmitter release	Protein growth factors
	Protein growth factors	
Altered electrical excitability; ion conductance	Neuroblasts	Ventricular myocytes
	Smooth muscle cells	
	Cerebrovascular myocytes	
Intracellular signaling		TNFα, adhesion
		PDGF, proliferation

1995; Tigyi *et al.*, 1995, 1996a,b; Postma *et al.*, 1996; Sakano *et al.*, 1996; Durante *et al.*, 1997; Kawa *et al.*, 1997; Oral *et al.*, 1997; Seewald *et al.*, 1997; Yatomi *et al.*, 1997; MacDonell *et al.*, 1998; Mathes *et al.*, 1998; Rodriguez-Fernandez and Rozengurt, 1998; Titievsky *et al.*, 1998; Xia *et al.*, 1998; Brocklyn *et al.*, 1999; Dubin *et al.*, 1999; Kranenburg *et al.*, 1999; Lee *et al.*, 1999a; Panetti *et al.*, 1999; Rizza *et al.*, 1999; Shahrestanifar *et al.*, 1999; Siess *et al.*, 1999; Zhang *et al.*, 1999). The third are linked to activation of ion fluxes that include Ca^{2+}, K^+, and Cl^-, which lead to contraction, secretion, and altered excitability in different cell types (Jalink *et al.*, 1994; Watsky, 1995; Postma *et al.*, 1996). Stimulation of proliferation of many different types of cells by both LPA and S1P, through a pertussis toxin-inhibitable mechanism, is the defining activity of PLGFs/LPLs. A complex set of LPL-initiated signaling pathways results in increases in intranuclear levels of the Ras-dependent ternary complex factor (TCF) and the Rho-dependent serum response factor (SRF), which together bind to and transcriptionally activate the serum response element (SRE) in promoters of many immediate-early response genes critical to cellular proliferation (Hill and Treisman, 1995; Hill *et al.*, 1995; Fromm *et al.*, 1997). It is presumed that Edg R transduction of LPL signals to SRE requires association with both $G_i/_o$ to recruit Ras and with $G_{12}/_{13}$ to engage Rho. Confirming evidence comes from the individual abilities of pertussis toxin inactivation of G_i and of *Clostridium botulinum* C3 ADP-ribosyltransferase inactivation of Rho to inhibit proliferative effects of LPLs and to exert greater suppression when applied in combination.

In addition to direct nuclear stimulation of cellular proliferation, LPLs also have indirect effects, which include increased secretion of autocrine protein growth factors, heightened expression of receptors for protein growth factors, and enhanced expression of plasma membrane-localized protein growth factors such as the heparin-binding epidermal growth factor (EGF)-like growth factor, which acts on EGF receptors of neighboring cells by a juxtacrine mechanism (Piazza *et al.*, 1995; Goetzl *et al.*, 1999b,d). In a few types of cells, where LPA elicits an increase in intracellular concentration of cyclic AMP, there is suppression of cellular proliferation (Tigyi *et al.*, 1994). The mechanisms whereby LPLs improve cellular survival are not fully understood, but include reduction in apoptosis. In the few types of cells for which mechanisms of suppression of apoptosis by LPLs have been elucidated, studies have detected both alterations in intracellular levels of effector proteins of the Bcl family and selective inhibition of activity of specific caspases. In T lymphocytes, the protective effects of LPLs are associated with increases in protective Bcl-2, decreases in apoptosis-promoting Bax, and inhibition of caspases 3, 6, and 7, but not 8 (Cuvillier *et al.*, 1996, 1998; Goetzl *et al.*, 1999a).

Cytoskeleton-based functional responses to LPLs include alterations in cellular morphology during differentiation, as typified by LPA-induced rounding of stellate periventricular neurons, and retraction of neurites evoked by LPA and S1P in postmitotic neurons (Table 2) (Tigyi and Miledi, 1992; Hecht *et al.*, 1996; Postma *et al.*, 1996; Tigyi *et al.*, 1996a,b; Brocklyn *et al.*, 1999; Kranenburg *et al.*, 1999). In such responses, stress fiber formation reflects changes in the state of the microfilament network. Activation of Rho-mediated signaling is responsible for the rearrangements of the actin cytoskeleton and actin-associated signaling molecules causing a loss of the differentiated phenotype. However, Rho-mediated signals also inhibit the signal transduction program of neuronal differentiation by inhibiting neurite outgrowth and the cessation of cell proliferation in response to treatments, which induce neuronal differentiation (Jalink *et al.*, 1994; Kozma *et al.*, 1997; Sebok *et al.*, 1999). Similarly to LPA, S1P affects vascular differentiation of endothelial cells, which involves the Rho-dependent formation of adherens junctions (Lee *et al.*, 1999b). LPA and S1P evoke focal adhesion kinase activity, activate cell surface adhesive proteins, and initiate assembly of a fibronectin matrix on cells (Ridley and Hall, 1992; Seufferlein *et al.*, 1996; Rodriguez-Fernandez and Rozengurt, 1998; Sakai *et al.*, 1998; Zhang *et al.*, 1999). Integrated expression of these responses mediates PLGF/LPL-elicited platelet aggregation and endothelial interactions with platelets and leukocytes. These and other related events are crucial to cellular chemotactic, secretory, and contractile responses to LPA and S1P. The principal current obstacle to better understanding of the mechanisms underlying cellular effects of LPLs is the absence of bioavailable and potent pharmacological agonists and antagonists specific for Edg Rs and other LPL Rs.

Regulatory molecules: Inhibitors and enhancers

A wide range of analogs and other variants of LPA and S1P have been synthesized in studies of the structural determinants of activity of the parent compounds (Bittman *et al.*, 1996; Liliom *et al.*, 1996; Lynch *et al.*, 1997; Fischer *et al.*, 1998), but most have the same undesirable physicochemical properties as

LPA and S1P, and none is a significantly more potent agonist or full antagonist of mammalian receptors. Limited applications of antireceptor antibodies, biochemical inhibitors of characteristic signaling pathways, and genetic approaches – such as overexpression of one receptor or antisense ablation of one receptor, have been useful in the early phases of research, but have not delineated use of individual receptors by cells nor been helpful for *in vivo* investigations.

Bioassays used

The basic bioassays have been summarized succinctly in published works (Spiegel and Merrill, 1996; Tigyi *et al.*, 1999).

IN VIVO BIOLOGICAL ACTIVITIES OF LIGANDS IN ANIMAL MODELS

Normal physiological roles

LPA and/or fluid-phase lysophospholipid precursors of LPA are elevated in at least four different clinical settings (**Table 3**): (1) acute lung diseases, such as *adult respiratory distress syndrome* (ARDS) and acute inflammatory exacerbations of chronic lung diseases, such as *asthma*, (2) surface epithelial cell injury, as in transcorneal freezing or cutaneous *burns*, (3) certain malignancies of which *ovarian cancer* is the most extensively analyzed, and (4) in CSF following *subarachnoid hemorrhage* (Yakubu *et al.*, 1997). Of these conditions, animal models have been established or adapted for studies of the roles of lysophospholipids in lung and ocular tissue trauma and inflammation.

Initiation of lung inflammation in guinea pigs by intratracheal administration of LPS induced secretion of type II secretory phospholipase A_2 ($sPLA_2$) into bronchoalveolar fluid and accompanying 3- to 10-fold increases in the concentrations of palmitic acid, total free fatty acids (FFAs), and lyso-phosphatidylcholine (lyso-PC) (Chilton *et al.*, 1996; Arbibe *et al.*, 1998). A specific inhibitor of $sPLA_2$ reduced by a mean of 60% the increases in levels of FFAs and lyso-PC evoked by LPS. Similar increases in the concentrations of FFAs and lyso-PC were attained by administration of guinea pig recombinant $sPLA_2$, in parallel with major decreases in surfactant content of phospholipids (Arbibe *et al.*, 1998). The capacity of lysophospholipase D in lung tissues to convert lyso-PC to LPA is suggested to be one source of increased LPA in pulmonary secretions of injured or inflamed lungs, but was not demonstrated directly. LPA and its active variants cyclic PA and alkenyl-GP and lysophosphatidylserine were identified at biologically active concentrations in aqueous humor and lacrimal gland fluid from rabbit eyes (Liliom *et al.*, 1998). The concentrations of LPA and its homologs were increased after corneal injury to levels which stimulated proliferation of keratinocytes isolated from uninjured rabbit corneas, suggesting a role in normal *wound healing*.

Knockout mouse phenotypes

These are limited to a receptor knockout (see chapter on Lysophospholipid growth factor receptors).

Interactions with cytokine network

LPLs modify secretion of some cytokine growth factors as noted above.

Table 3 Pathophysiological contributions

Model or disease	Site	LPL	Contribution
Rabbit corneal injury	Aqueous humor	LPA, LPA isomers	*Wound healing*
Human lung injury	BAL	LPA, LPC, other PLs	Wound healing
Human *atherosclerosis*	Vascular lesions	LPA	Platelet/endothelial activation
Human *ovarian cancer*	Tumor/*metastases*	LPA	*Tumor growth*, spread
			Increased vascular permeability

BAL, bronchoalveolar lavage fluid.

PATHOPHYSIOLOGICAL ROLES IN NORMAL HUMANS AND DISEASE STATES AND DIAGNOSTIC UTILITY

Normal levels and effects

The plasma concentrations of LPA and S1P are mid-nanomolar and up to micromolar, respectively, and serum concentrations of both are micromolar.

Role in experiments of nature and disease states

Elevated concentrations of LPA, lyso-PC, and some other phospholipids have been detected in lesional fluids of several inflammatory and neoplastic diseases (Table 3). However, only in ovarian carcinoma and possibly other gynecologic cancers have tissue and plasma levels of LPA been increased so consistently as to suggest pathogenetic roles and even utility as a biochemical marker of these malignancies (Xu *et al.*, 1998). The ability of LPA to stimulate increased expression by *ovarian cancer* cells of adhesive proteins characteristic of the neoplastic state, such as vascular endothelial growth factor (VEGF) (Hu *et al.*, 2000), transcellular migration (Imamura *et al.*, 1993), and proliferation (Goetzl *et al.*, 1999e), without effects on normal ovarian surface epithelial cells, has contributed to the authenticity of suggestions that plasma levels of LPA represent a useful marker for even early stages of ovarian cancer. Any role of LPA in ovarian and other cancers remains to be proven in further analyses of mechanisms and much larger clinical studies, involving the effects of different forms of treatment. However, it appears certain that ovarian cancer cells do produce LPA in amounts far exceeding those of any other form of cancer so far examined *in vitro*. Further, ovarian cancer cells express Edg Rs for LPA qualitatively different from those detected on normal ovarian cells (see chapter on Lysophospholipid growth factor receptors).

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Lysophospholipid Growth Factor Receptors

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SUMMARY

The endothelial differentiation gene-encoded G protein-coupled receptors (Edg Rs) Edg-1, -3, -5, and -8 bind sphingosine 1-phosphate (S1P) and Edg-2, -4, and -7 bind lysophosphatidic acid (LPA), resulting in transduction of cellular signals for proliferation, increased survival, reduced apoptosis, adherence, migration, secretion, and other specific functions. The diversity of signals emanating from each Edg R is in part attributable to coupling to multiple G proteins. One or more Edg Rs are expressed prominently in the cardiovascular, pulmonary, nervous, endocrine, genitourinary, hematological, and immune systems. Expression of Edg Rs is regulated by developmental and postdevelopmental physiological events. The levels of Edg Rs increase in distinctive patterns in many diseases, as exemplified by high levels of Edg-4 in *ovarian cancer*, whereas none is detected in normal ovarian surface epithelial cells. Although several analogs of LPA and S1P have altered agonist or antagonist activities, potent pharmacological agents have not been identified to date.

BACKGROUND

LPA, S1P, sphingosylphosphorylcholine and other structurally related lysophospholipid (LPL) growth factors have major effects on many different types of cells, which include initiation and regulation of cellular proliferation, enhancement of survival, suppression of apoptosis, promotion of differentiation, and stimulation of diverse cytoskeleton-based functions (Moolenaar, 1995; Tokomura, 1995; Spiegel and Merrill, 1996; Goetzl and An, 1998). LPLs are generated enzymatically from precursors stored in cell membranes and are secreted principally by platelets, macrophages, some other types of leukocytes, epithelial cells, and some cancer cells. The amounts secreted are sufficient to establish micromolar concentrations of LPA and S1P in plasma and S1P in plasma normally and in extracellular fluids during tissue reactions. Extracellular LPA and S1P are both almost entirely bound by serum proteins (Tigyi and Miledi, 1992; Thumser *et al.*, 1994). LPLs resemble protein growth factors in their ability to alter transcriptional activities of growth-related genes, amplify direct stimulation of cellular proliferation by recruiting other growth factor systems, and evoke many cellular responses other than proliferation and proliferation-related events (An *et al.*, 1998c; Goetzl and An, 1998).

Discovery

The LPLs and protein growth factors differ principally in their respective uses of G protein-coupled receptors (GPCRs) and protein tyrosine kinase receptors. LPA and S1P bind specifically to different members of a family of GPCRs, which are encoded by endothelial differentiation genes (Edgs) and thus are designated tentatively as Edg Rs (Hla and Maciag, 1990; MacLennan *et al.*, 1994; Yamaguchi *et al.*, 1996; Chun *et al.*, 1999). Edg-1 was discovered initially as a GPCR of unknown ligand specificity, which was expressed by endothelial cells only after

differentiation (Hla and Maciag, 1990). Edg-2 was first found on proliferating neurons in the periventricular zone of developing embryonic mice (Hecht *et al.*, 1996). LPA evoked shape changes and proliferation in these Edg-2 R-expressing neurons, and both their Edg-2 Rs and the responses to LPA disappeared after completion of CNS development. The primary determinants of cellular responses to LPLs are the generative and biodegradative events, which establish steady-state concentrations of each protein-bound LPL at cell surfaces, and the relative frequency of expression of each Edg R.

Alternative names

There are no alternative names for the Edg receptor family, but individual receptors have been called by other terms, such as ventricular zone gene 1 (vzg-1)-encoded receptor for Edg-2 (Hecht *et al.*, 1996).

Structure

Two subfamilies of the Edg Rs have been distinguished based on their degree of amino acid sequence identity and preferred LPL ligand (**Table 1**) (Goetzl and An, 1998; Chun *et al.*, 1999). The first encompasses Edg-1, -3, -5, and -8, which are 45–60% identical in amino acid sequences and bind S1P with high affinity (Hla and Maciag, 1990; MacLennan *et al.*, 1994; Yamaguchi *et al.*, 1996; An *et al.*, 1997b; Lee *et al.*, 1998a,b; Glickman *et al.*, 1999). The second includes Edg-2, -4, and -7, which are 48–54% identical in amino acid sequences and bind LPA with high specificity and affinity (Hecht *et al.*, 1996; An *et al.*, 1997a, 1998a; Bandoh *et al.*, 1999). Mechanisms of regulation of expression, G protein associations, predominant signaling pathways, pattern of tissue representation, and involvement in human diseases are now being defined for each Edg R.

Main activities and pathophysiological roles

The principal roles of Edg Rs are to bind LPA and/or S1P with high affinity and specificity, and to transduce G protein-dependent signals to cellular proliferation, survival, and functions.

GENE

The genomic structures of Edg-1, -3, and -5 differ fundamentally from those of Edg-2 and -4. The over-45 kb murine Edg-2 gene was successfully defined and shown to be composed of at least five separate exons on chromosome 4 (Contos and Chun, 1998). Divergent cDNA sequences are located upstream from a single common exon sequence, which encodes most of the open reading frame of Edg-2 and ends at an intron in the middle of transmembrane domain VI. In contrast, murine Edg-1 has only two exons, with a

Table 1 Encoding genes and structures of Edg LPA and S1P receptors

Human receptor	Ligand	Chromosomal location (human/mouse)	Protein size (aa no.)	G protein coupling	Signaling		
					A	C	E
Edg-1	S1P	1p21.1–3	381	i	I	S	S
Edg-3	S1P	9q22.1–2	378	i, q, 12/13	I	S	S
Edg-5	S1P	19p13.2/9	354	i, q, 12/13	I	S	S
Edg-8	S1P	ND	400				
Edg-6	ND	19p13.3	384	–	–	–	–
Edg-2	LPA	ND/4 (6)	364	i, 12/13	I	S	N/S
Edg-4	LPA	19p12	351 (382)[a]	i, q, 12/13	I	S	S
Edg-7	LPA	ND	353	q, s	S	S	N/S

A, adenylyl cyclase; C, increase in $[Ca^{2+}]_i$; E, ERK activity; ND, not determined; I, inhibit; S, stimulate; N, no effect; –, insufficient data; N/S, no effect in some cells, stimulation in other cells.

[a]The number of amino acids in the wild type is 351 and in the mutant is 382.

single coding exon, an intron separating the transcriptional start site from the open reading frame, and no intron in transmembrane domain VI (Liu and Hla, 1997). Genes encoding several other Edg Rs have very recently been defined structurally or sequenced by the Human Genome Project (Table 1). A recently recognized mutant of Edg-4 R results from a deletion of a single base near the 3′ end of the open reading frame, which leads to a frameshift, loss of the termination codon, and replacement of the four amino acid C-terminus of the wild-type Edg-4 with a completely different 35 amino acid peptide (Table 1). No promoter elements of any Edg R have been analyzed further as yet.

Accession numbers

Edg-1: AF022137, M312104
Edg-3: AF022139, X83864
Edg-5: AF034780
Edg-6: AJ000479
Edg-2: U80811
Edg-4: AF011466
Edg-7: AF127138

Chromosome location and linkages

See Table 1.

There is approximately a 26–28% homology in sequence between Edg Rs and some cannabinoid GPCRs.

PROTEIN

Description of protein

The Edg-1 to Edg-8 genes encode distinct but related seven transmembrane domain GPCRs (Table 1). Based on amino acid sequence identity, Edg-1, -3, -5, and -8 belong to one structural cluster and Edg-2, -4, and -7 are members of a second structural cluster. The amino acid sequence of Edg-6 lies between those of the two major clusters (Graler *et al.*, 1998). Edg Rs share other structural features which have not as yet been definitively linked to function. The *N*-linked glycosylation sites of the N-terminus and multiple potential sites of phosphorylation in intracellular regions are typical of all GPCRs and are preserved in the Edg Rs. In contrast, the disulfide bond most often formed between cysteines in the first and second extracellular loops in other GPCRs is most often formed between the second and third extracellular loops of Edg Rs. In Edg-4 R, an alanine replaces the proline typical of the seventh transmembrane domain sequence NPXXY of other GPCRs. An Src homology 2 (SH2) segment exists in the intracellular face of Edg-5, but has not been shown to express characteristic functions. The mRNAs encoding some of the Edg Rs have the AU-rich sequence AUUUA in their 3′ untranslated region, which is an mRNA-stabilizing structure typical of growth-related immediate-early genes. A GPCR originally reported as an orphan (An *et al.*, 1995), has been proven to bind the LPL sphingosylphosphorylcholine with high specificity and affinity, resulting in the expected intracellular signals (Xu *et al.*, 2000).

Distinctive patterns of tissue distribution of each Edg R have been mapped principally by semiquantitative PCR techniques, northern blots, *in situ* hybridization and western blots with monoclonal antibodies to multiple substituent peptides (An *et al.*, 1997a,b, 1998a; Hecht *et al.*, 1996; Liu and Hla, 1997; MacLennan *et al.*, 1997; Weiner *et al.*, 1998) (Table 2). Another type of GPCR has been identified on *Xenopus* oocytes and some rodent cells, which is specific for LPA but structurally unrelated to Edg Rs (Guo *et al.*, 1996).

Cell types and tissues expressing the receptor

These are listed in **Table 2**.

SIGNAL TRANSDUCTION

Each of the Edg-1 to -8 receptors usually couples to multiple G proteins, which results in concurrent initiation of diverse signaling pathways that may be amplified by crosstalk between these pathways (Goodemote *et al.*, 1995; Brindley *et al.*, 1996; Lee *et al.*, 1996; van Koppen *et al.*, 1996; An *et al.*, 1998b; Erickson *et al.*, 1998; Fukushima *et al.*, 1998; Okamota *et al.*, 1998; Zondag *et al.*, 1998; Windh *et al.*, 1999). The association of each Edg R with G_i leads to striking ligand-induced enhancement of the Ras/extracellular signal-regulated kinase (ERK) pathway central to LPA and S1P activation of cellular proliferation (Table 1). The $\beta\gamma$ subunits of G_i may couple ERKs to Edg Rs, whereas the α chain of G_i mediates the defining inhibition of adenylyl cyclase. The biochemical prerequisites for G_i recruitment of ERKs include an Src-related tyrosine kinase, Pyk 2 kinase, and probably PI-3 kinase, based on the

Table 2 Human Edg receptor tissue distribution and abnormalities in disease states

	Tissue distribution	Expression/mutations in diseases
Edg-1	Endothelial cells, many other types of cells	
Edg-3	Heart, many other types of cells	Increased in *breast cancer*
Edg-5	Heart, embryonic nervous system, many other types of cells	
Edg-8	Brain	
Edg-6	Lymphoid tissues, leukocytes, lung	
Edg-2	Embryonic nervous system, many other types of cells	
Edg-4	Leukocytes, testes, many other types of cells	Increased in breast cancer, increased in *ovarian cancer* (mutant form)
Edg-7	Pancreas, heart, prostate, testes, lung, ovary	

ability of selective inhibitors of PI-3 kinase to suppress LPA and S1P induction of ERK activity.

Edg-3, -4, -5, and -7 receptors couple with and activate G_q, resulting in sequential stimulation of phospholipase C, release of diacylglycerol leading to enhancement of protein kinase C activity and of inositol trisphosphate (IP_3), which consequently elicits pertussis toxin-insensitive increases in $[Ca^{2+}]_i$. In contrast, G_i mediates the pertussis toxin-sensitive part of LPA- and S1P-evoked increases in $[Ca^{2+}]_i$ transduced by Edg-3, -4, and -5, and the principal effect transduced by Edg-1 and -2 in some types of cells through recruitment of phospholipase C. The proposed capacity of S1P to act as intracellular messenger and thereby mobilize Ca^{2+} directly, without binding to a cell surface receptor, is independent of IP_3 and dependent on sphingosine kinase activity in some cells, but the cellular target protein for this action of S1P remains to be identified (Spiegel and Merrill, 1996; Kohama *et al.*, 1998; Melendez *et al.*, 1998).

LPA and S1P also evoke cellular proliferation and cytoskeleton-dependent functions through Rho-mediated pathways, which are initiated by engagement of any of the Edg Rs (Moolenaar, 1995; Spiegel and Milstien, 1995; Fromm *et al.*, 1997) (Table 1). In some cellular settings, Edg-1 may not couple to Rho. The elicitation of SRF transcriptional activity, regulation of cellular pH, and induction of formation of actin stress fibers and focal adhesion complexes by LPA and S1P are all Rho-mediated through $G\alpha_{12/13}$ or, in rare circumstances, through $G\alpha_q$ (Hill and Treisman, 1995; Hill *et al.*, 1995; Fromm *et al.*, 1997). The GTPase-activating factor Lsc/p115RhoGEF links $G\alpha_{13}$ with Rho and enables Rho, which in turn activates serine/threonine kinases, phospholipase D, SRF, PI-3 kinase, p125 focal adhesion kinase, and myosin light chain phosphatase (Hart *et al.*, 1998). Many effects of LPA and S1P on complex cellular responses involve integrated engagement of multiple signaling pathways.

DOWNSTREAM GENE ACTIVATION

Transcription factors activated

Transcriptional activation of SRE by LPA and S1P suggested the possibility that recruitment of growth-related immediate-early genes with SRE in their promoter, such as c-fos and others critical for proliferative responses, might be an important mechanism mediating growth effects of the LPLs. In some cancer cells, LPA and S1P also upregulate expression of autocrine protein growth factors and their receptors through transcriptional mechanisms, which remain to be defined (Goetzl *et al.*, 1999a,b).

BIOLOGICAL CONSEQUENCES OF ACTIVATING OR INHIBITING RECEPTOR AND PATHOPHYSIOLOGY

All of the effects of LPA and S1P described in the chapter on the lysophospholipid growth factors are presumed to be transduced by the Edg Rs and related GPCRs. The LPLs are often acting in tissues responding to other cytokines and mediators, and it is not known how these factors influence expression and functions of receptors for LPLs. The absence of

native inactivating genetic defects for any of the receptors and of potent pharmacological agents presently precludes specific assignment of any of the LPL effects to one type of receptor. Some native variants of LPA and synthetic modifications of LPA or S1P have interacted with Edg Rs as partial agonists or antagonists (Bittman *et al.*, 1996; Liliom *et al.*, 1996; Lynch *et al.*, 1997; Fischer *et al.*, 1998; Tigyi *et al.*, 1999). These have increased our understanding of cellular events, but are not considered to be useful for *in vivo* investigations.

Further investigations of the recently described Edg-2 knockout mice (Contos *et al.*, 2000) and development of agonists and antagonists may soon permit useful assessments of the roles of each receptor. Early reports of increases in expression or *de novo* appearance of one or more Edg Rs in *malignant neoplasms*, when contrasted with the patterns of expression in nonmalignant cells of the same lineage, are of interest, but difficult to interpret currently (Goetzl *et al.*, 1999a,b). The most striking finding is the appearance of Edg-4 in *ovarian cancer*, without expression in normal or virally transformed ovarian surface epithelial cells (Goetzl *et al.*, 1999b). At least one ovarian cancer expressed a mutant Edg-4 characterized by increased length of the cytoplasmic tail. Ongoing studies are directed to elucidation of this mechanism and identification of additional mutants in ovarian and other neoplasms.

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PROSTAGLANDINS & other LIPID MEDIATORS

Pleiotypic mechanisms of cellular responses to biologically active lysophospholipids

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Abstract

The activities of cell-derived lysophospholipid (LPL) growth factors on cellular proliferation and a range of proliferation-independent functions are regulated at multiple levels. This section focuses first on the capacity of the actin-severing protein gelsolin to bind lysophosphatidic acid (LPA), but not sphingosine 1-phosphate (S1P), and either sequester LPA or present it to responsive cells. Expression of members of the family of endothelial differentiation gene-encoded G protein-coupled receptors (Edg Rs) for LPLs is controlled developmentally and by cell-activating stimuli. Edg R transduction of cellular effects of LPLs involves both direct actions on target cells and induction of generation of proteins with relevant actions capable of amplifying or diminishing primary direct effects of LPLs. These general mechanisms are evident in Edg R mediation of proliferation, cytokine secretion and suppression of apoptosis. The availability of functionally-active anti-Edg R antibodies and Edg R-specific pharmacological probes, establishment of Edg R transgenes and gene knockouts, and identification of natural genetic anomalies of LPL metabolism and recognition by Edg Rs will permit elucidation of the *in vivo* activities of LPA and S1P normally and in disease states.

Keywords: Growth factors; Cytokines; Gelsolin; Apoptosis; Receptors

1. Introduction

There have been astounding advances in our understanding of the biological roles of two major subfamilies of lysophospholipid (LPL) mediators in the past four years. Biologically active LPLs are generated by many types of mammalian cells and they have protean

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capacities to affect growth and functions of diverse cells in multiple organ systems [1–5]. Lysophosphatidic acid (LPA) is the most prominent member of the lysoglycerol-containing phospholipid subfamily, which predominates quantitatively among lipid structural components of cellular membranes. Sphingosine-1-phosphate (S1P) is a highly-active lysosphingophospholipid, which is structurally and functionally related to LPA. The subfamily of cellular lysosphingophospholipids are quantitatively diminutive in contrast to the subfamily of lysoglycerophospholipids, but exhibit great structural complexity and have a range of biological effects similar to those of lysoglycerophospholipids. Alkenyl- and cyclic-variants of LPA, and sphingosylphosphorylcholine are other naturally-occurring LPLs which exhibit many cellular effects. The LPLs of both subfamilies are related also by being products of metabolism of cellular membrane phospholipids, increasing in concentration transiently in relation to cellular responses, requiring carrier proteins for cellular presentation, moving and interacting with proteins in cellular membranes, and potently influencing cells through one or more subfamilies of G protein-coupled receptors (GPCRs) [6–10]. Our knowledge of every aspect of the cellular generation, recognition and effects of LPLs has progressed recently through discoveries of distinctive biosynthetic and metabolic pathways and the definition of structures and signaling pathways of the many members of a novel subfamily of endothelial differentiation gene (edg)-encoded GPCRs (Edg Rs) dedicated to LPLs.

2. Diversity of lysophospholipid cellular sources and binding proteins

The nature and amounts of LPLs produced by multi-enzymatic pathways are determined by the origin, basic type, proliferative state, and level of functional activation of the cells [1–5]. Platelets, macrophages, other leukocytes, some epithelial cells and some tumor cells are major sources of both LPA and S1P. Although fibroblasts responding to protein growth factors produce LPA and S1P, only small amounts appear to be released into extracellular fluids. LPA also is synthesized at interfaces between cellular plasma membranes and extracellular fluid both by secretory phospholipase A2 hydrolysis of PA exposed on microvesicles shed from activated platelets and leukocytes, and by phospholipase D cleavage of leukocyte-surface lysophosphatidylcholine. LPL generation from non-malignant cells is initiated by physical perturbation or by stimulation of plasma membrane receptors or other surface proteins, which activate critical controlling enzymes such as phospholipases and sphingosine kinase. In contrast, tumor cells may secrete LPLs spontaneously, which was described first for LPA production by ovarian cancers. LPA and S1P were identified recently in mildly-oxidized low density lipoproteins of the lipid-rich core in atherosclerotic plaques, where they may contribute to proliferation of vascular smooth muscle cells, as well as platelet and endothelial activation. Characterization of cellular production of LPLs under physiological conditions is limited to only a few examples.

Platelet adhesion to vascular surfaces and homotypic aggregation induced by thrombin result in generation of both LPA and S1P in quantities sufficient to raise effective LPL concentrations up to 1 to 5 μM [11,12]. Alpha$_2$-adrenergic stimulation of adipocytes provokes release of LPA at concentrations capable of inducing proliferation, spreading and maturation of preadipocytes [13]. The elevated level of production of LPA by ovarian

carcinoma cells leads to concentrations in ascites of up to 20 μM and even up to 10-fold higher than normal in plasma of patients, in direct relationships with the stage of disease and tumor burden [14].

The critical functional role of fluid-phase protein-binding for solubility, sequestration and cellular delivery of LPLs has been demonstrated in many model systems [1–5]. Most secreted LPA and S1P are bound by serum albumin, which is a high-capacity and low-affinity (Kd = 360 nM for LPA) reservoir capable of delivering LPLs to all known target cells. Two quite independent investigations recently documented the capacity of LPA to interact functionally with plasma gelsolin [15,16]. The results of one showed that LPA suppresses the actin-severing activity of gelsolin in a manner similar to L-alpha-phosphatidylinositol-4,5-diphosphate (PIP2) by apparently binding to the same two sites [15] and the other that high-affinity binding of LPA by normal plasma concentrations of gelsolin reduced its cellular effects, whereas much lower concentrations of gelsolin led to more efficient cellular delivery of LPA than serum albumin [16]. Equilibrium binding of ^{3}H-LPA by human purified plasma gelsolin and human recombinant gelsolin were characterized by a valence of up to 1.8 and respective mean Kd values of 6.2 nM and 32 nM, as contrasted with 357 nM for fatty acid-free bovine serum albumin.

Characteristics of the binding curves and gelsolin concentration-dependence of effects on cellular activities of LPA suggested binding site cooperativity, such that gelsolin levels of or lower than 10% of that in normal plasma delivered LPA to cells very effectively, whereas higher concentrations reduced LPA activity relative to that seen with serum albumin [16]. Initial studies of the functional consequences of binding of LPA by gelsolin were conducted in rat cardiac myocytes (RCMs), which were washed thoroughly prior to stimulation to remove any endogenous gelsolin. In the absence of protein, nuclear signaling in RCMs transfected with an SRE-luciferase reporter was observed luminometrically only with 10^{-6} M LPA, whereas there were significant reports at 10^{-7} M and 10^{-6} M LPA with 100 μg/ml of fatty acid-free BSA or 30 μg/ml gelsolin, and 10^{-9} M to 10^{-6} M LPA with 3 μg/ml gelsolin. Stimulation of RCM hypertrophy, as assessed by increased incorporation of ^{3}H-leucine into proteins, similarly was stimulated significantly more by 10^{-8} M to 10^{-6} M LPA in 3 μg/ml and 10 μg/ml of gelsolin than 100 μg/ml of fatty acid-free serum albumin or protein-free medium.

That gelsolin is the active protein was supported by finding that human recombinant gelsolin and human purified plasma gelsolin exhibited similar concentration-activity relationships for cellular signaling and functional effects [16]. The role of gelsolin was confirmed further by demonstrating inhibition by anti-gelsolin antibody of signaling of cardiac myocytes by LPA with gelsolin, but not with serum albumin. The lack of alteration of LPA-like cellular effects of anti-Edg-4 R antibody and of S1P by gelsolin showed that gelsolin acted directly on LPA, rather than on an element of Edg R mediation or cellular transduction. The possibility of LPA binding to PIP2 sites of gelsolin was validated by characteristics of competitive interactions between LPA and both PIP2 and gelsolin substituent peptides constituting the known PIP2 binding sites in relation to cellular responses, as well as fluid-phase binding [17].

Although our understanding of the role of gelsolin as a carrier and cellular presenter of LPA is still developing, it is possible to propose a tentative model (Fig. 1). For cells which

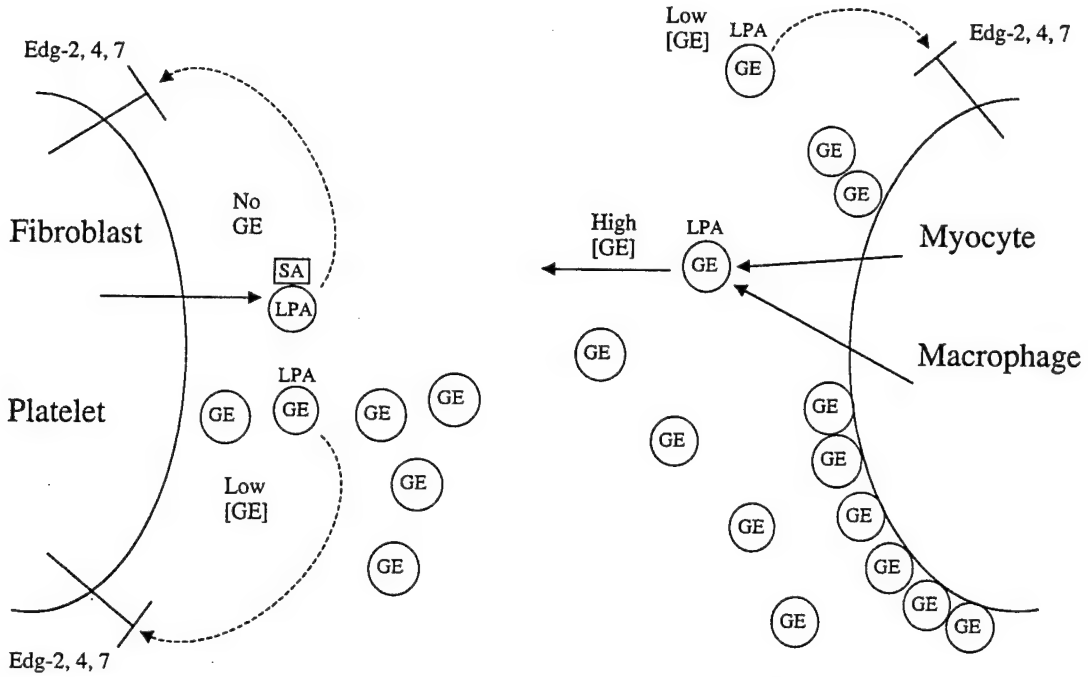

Fig. 1. Trapping, sequestration and cellular presentation of LPA by secreted gelsolin. SA = serum albumin, GE = gelsolin, T = Edg R for LPA; solid line = release or diffusion and dashed line = receptor stimulation.

produce gelsolin in functionally-relevant quantities, such as myocytes and macrophages, high local concentrations of gelsolin would minimize autocrine actions of LPA, as well as actions of LPA from other sources by sequestration and diffusional dissipation. At lower concentrations, gelsolin may present less avidly-bound LPA to Edg Rs. For cells which secrete little or no gelsolin and generate high or low concentrations of LPA, such as platelets and fibroblasts respectively, serum albumin may be the principal carrier and cellular presenter or this function may be shared with low levels of gelsolin from other cells. Other proteins capable of binding S1P and other bioactive lysosphingolipids may exist and could have functions analogous to gelsolin for LPA.

3. Cellular G protein-coupled receptors for lysophospholipids

As the structures of Edg Rs and other GPCRs for LPLs of both major families have been reviewed recently and will be discussed in several other sections of this volume, the present comments will focus on unique features of cell- and tissue-specific distribution of Edg Rs, and results of early analyses of developmental and activation-induced regulation of expression of Edg Rs. The initial realization that many different types of mammalian cells express two or more types of Edg Rs prompted searches for Edg R-null cells, which could be used for investigations of the biochemical and cellular properties of individual Edg Rs introduced by transfection. These surveys and related studies led to three observations of novel biolog-

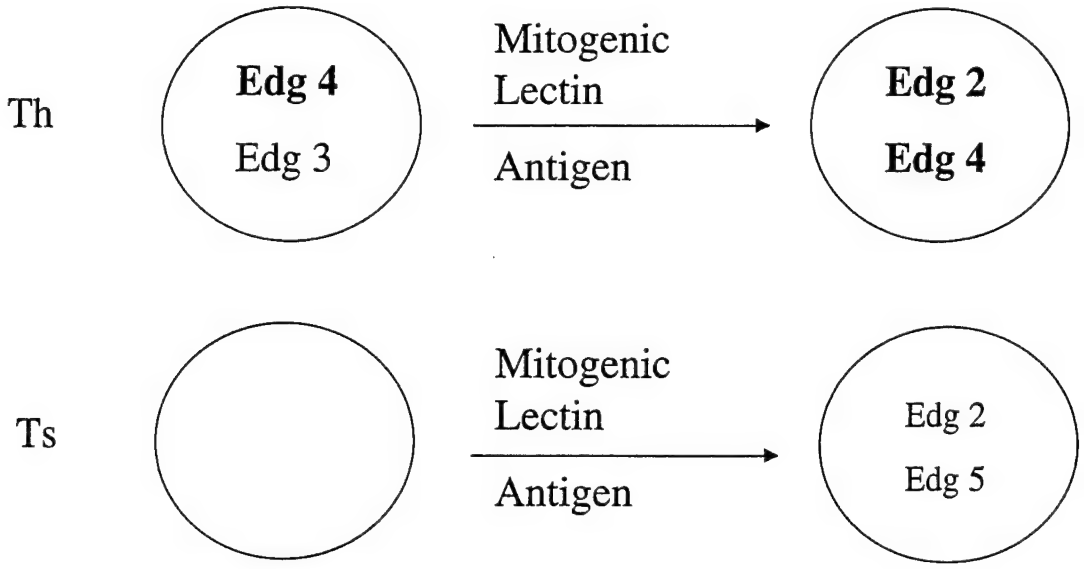

Fig. 2. Effects of activation of T helper lymphocyte on expression of Edg receptors. Th = helper T cell, Ts = suppressor T cell. The weight of characters depicting each Edg receptor signifies its relative frequency of representation.

ical properties of Edg Rs. The **first** was that differentiation and maturation of some types of hematopoietic and immune cells resulted in substantial modification of their complement of Edg Rs. Induction of differentiation of human promyelocytic leukemia cells of the HL-60 line by three distinct stimuli resulted in suppression of expression of functional Edg-3 Rs, as shown by a striking reduction in S1P-evoked increases in $[Ca^{++}]i$ [18]. Mouse early thymocytes express Edg-3 and -5 Rs, detected by reverse transcription-polymerase chain reaction (RT-PCR) semi-quantification of mRNA and by Western blots, which nearly disappear after exposure of the thymocytes to antigen and antigen-presenting cells or other stimuli of differentiation. Edg-4 and -6 become the predominant LPL receptors on mature T helper cells after the earliest subdivision into T helper/inducer and T suppressor/cytotoxic sets, with only minor residual representation of Edg-3 on T helper/inducer cells and no detectable Edg Rs on T suppressor/cytotoxic cells, as determined from analyses of human blood T cells (Fig. 2) [19,20]. Mitogen- or antigen-elicited activation of human blood-derived T helper cells results in loss of Edg-3 R, a slight decrease in Edg-4 and the appearance of a high level of Edg-2 R, according to results of RT-PCR analyses and Western blots [20]. These alterations in profiles of Edg Rs are accompanied by the expected changes in responses to LPA and S1P.

The **second** observation was that malignant transformation of some types of cells results in the appearance and often predominance of one or more Edg Rs not expressed by the equivalent non-malignant cell. Numerous human ovarian cancer cell lines express high levels of Edg-4 R not detectable in either primary cultures of human normal ovarian surface epithelial cells or immortalized lines of human normal ovarian surface epithelial cells [21]. In other tumors, malignant transformation results in quantitative changes in levels of

expression of more than one Edg R. For example, several lines of human breast cancer cells have higher levels of Edg-3 and -5, and a lower level of Edg-2 than human normal breast epithelial cells in primary cultures [22]. It remains to be determined if such differences contribute in any way to pathogenesis or even may serve as functional markers of some aspect of cancer biology.

The **third** was an apparent lack of variants of Edg Rs attributable to mutations or alternative splicing, compared to other families of GPCRs of similar size and complexity, such as those for some neuropeptides and for prostanoids. One alternative form of human Edg-4 R was discovered in ovarian cancer cells and found to result from loss of one G, which caused a frame-shift disrupting the termination codon and leading to carboxyl-terminal elongation by 33 amino acids [9]. As extracellular and cytoplasmic loops of Edg-4 are small, it is assumed that many transductional factors dock on the cytoplasmic carboxyl-terminal tail, as for other structurally similar GPCRs. Thus it was not surprising to discover that the longer Edg-4 R is a modest gain-of-function mutant.

4. Multiplicity of cellular functional responses to lysophospholipids: shared mechanisms

LPLs influence nearly every element of cellular function from proliferation to synthetic activities to distinctive effector functions to apoptosis. Two central concepts which have emerged from studies of cellular functional responses to LPLs are: 1) Edg R mediation of an effect of an LPL often involves direct and indirect mechanisms, and 2) Different Edg Rs specific for the same LPL may transduce distinct or even opposite effects (Fig. 3, left side). Edg R transduction of LPL stimulation of proliferation of breast and ovarian cancer cells involves first direct signaling through the SRE in promoters of many immediate-early growth-related genes, and then stimulation of production, secretion, and effects of several different endogenously-derived protein growth factors. These and related earlier observations in keratinocytes suggested the first distinctive biological aspect of Edg R mediation of growth (Fig. 3, left side). LPL-stimulated breast and ovarian cancer cells both report great increases in SRE-luciferase activity, which attains a plateau within 4–5 hr. LPL-stimulated breast and ovarian cancer cells then secrete higher levels of type 2 insulin-like growth factor (IGF-II), which reaches a peak after 36–48 hr [21,22]. With the same time-course, LPL-stimulated ovarian cancer cells, but not breast cancer cells, secrete much higher levels of vascular endothelial growth factor (VEGF) which mediates altered permeability as well as cell proliferation [23]. That this indirect later pathway dominates after 24 hr of LPL stimulation was demonstrated by achieving over 90% suppression of S1P-elicited breast cancer cell proliferation with a neutralizing antibody to the type 1 receptor for IGF-II (IGFR 1). Similar recruitment of endogenous protein growth factors has been demonstrated in human T lymphoblastoma cells, where both LPA and S1P elicit increases in membrane-associated heparin-binding epidermal growth factor-like growth factor through a ras-dependent mechanism [24].

Some lines of human ovarian cancer cells have high levels of Edg-2, as well as of the more distinctive Edg-4 type of LPA receptor. It had been shown earlier that LPA could suppress

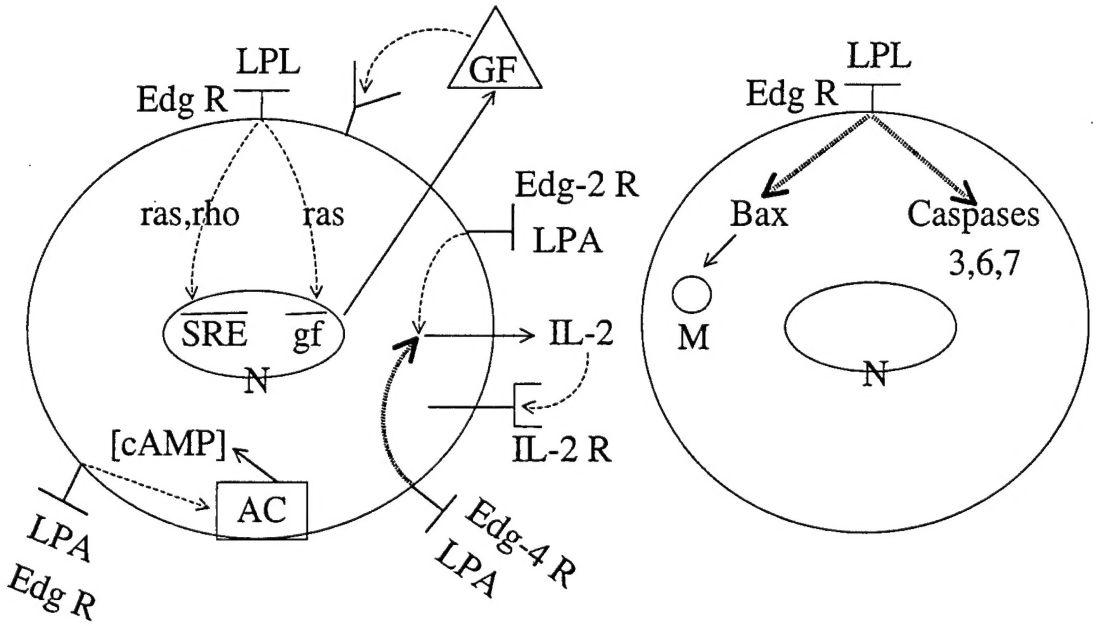

Fig. 3. Edg receptor transduction of effects of LPLs on proliferation and apoptosis. SRE = serum response element in the promoter of immediate-early growth-related response genes, gf = growth factor-encoding gene, GF = protein growth factor, IL-2 = interleukin-2, AC = adenylyl cyclase, [cAMP] = intracellular concentration of cyclic AMP, N = nucleus, M = mitochondria. Solid line = production or secretion, dashed line = stimulation, wavy or double dotted line = inhibition or suppression.

proliferation of some types of cells in association with increases in [cAMP]i [25]. On this background, the second distinctive biological aspect of Edg R signaling of growth was recognized from observations that the proliferative responses of high Edg-2 R-bearing ovarian cancer cells to LPA were uniformly less than those of low Edg-2 R-bearing lines [26]. That this differential responsiveness was attributable to transduction of a proliferation-inhibiting signal from LPA-occupied Edg-2 Rs was suggested by the stimulatory effect of anti-Edg-4 R monoclonal antibody on proliferation of ovarian cancer cells and by the suppressive effect of anti-Edg-2 R monoclonal antibody. Similar opposing activities of the two LPA Rs also was observed recently in human blood T helper cells stimulated to produce IL-2 by activation of their T cell receptor with adherent anti-CD3 plus anti-CD28 antibodies [20]. Freshly isolated T helper cells express predominantly Edg-4 R and either LPA or anti-Edg-4 R antibody decreases secretion of IL-2 [20]. In contrast, mitogen-activated T helper cells express more Edg-2 R than Edg-4 R and in them LPA or anti-Edg-2 R antibody increases secretion of IL-2 [27]. The biochemical and cellular mechanisms accounting for opposite effects of Edg-2 and Edg-4 Rs are not yet known.

The Edg Rs mediate at least two highly specific actions which suppress apoptosis initiated by diverse stimuli (Fig. 3, right side). The first is reduction in the cellular concentration of the pro-apoptotic protein Bax, which was quantified in a human T lymphoblastoma cell line by Western blots developed with two antibodies of different specificities [28]. Results of recent studies also have revealed Edg R transduction of decreases in apoptosis-associated

insertion of Bax into mitochondria of T lymphoblastoma cells. A second mechanism of inhibition of apoptosis identified in a line of leukemic human T cells is suppression of the activities of death caspases 3, 6 and 7, but not 8 [29]. As for the mechanisms of Edg R effects on cellular proliferation, both anti-apoptotic actions result from alteration in the levels or activities of critical control or effector proteins.

5. Specificity of cellular responses and adaptation to lysophospholipids

Edg R mediation of a particular response of any type of cell to an LPL is determined by many factors, of which the most important appear to be the natural range of functions of that cell, the density of expression of each type of Edg R, and the efficiency of coupling of cellular signaling pathways to each type of Edg R. For an LPL to stimulate chemotactic migration of a cell, for example, the target cell must be capable of migration, the density of the cognate Edg R must be sufficient to permit detection of a concentration gradient across the diameter of the cell, and the Edg R must transduce all signals which are integrated in the constitution of chemotaxis. These signals clearly require coupling of the engaged Edg Rs to multiple G proteins, involve changes in $[Ca^{++}]i$ and [cAMP]i, and would alter functions of the cell as diverse as adhesiveness, cytoskeletal structure, and secretion of proteases necessary for transmigration of basement membranes and other tissue structures. As efficiency of coupling of each Edg R to any G protein differs, this may be another example of an advantage of expression of several Edg Rs for the same LPL by one type of cell. Each transductional mechanism and component of the integrated response also is anticipated to be affected by signals from receptors for other co-elicited mediators on the responding cells. Thus specificity is determined by numerous factors external to the LPL-Edg R system, as well as those constituting the basic response mechanisms. Most of the basic and external determinants of specificity predicted to act in the LPL-Edg R system have not been elucidated thus far.

Many other aspects of Edg R mediation of cellular responses to LPLs also have not been approached experimentally. Essentially nothing is known of mechanisms of hypersensitization, accommodation and desensitization or of interactions with other growth factor and mediator systems. Ongoing studies have just begun to approach even the most straightforward elements of Edg R control of cellular responses, such as relationships between receptor density and response magnitude or cross-talk between different Edg Rs.

6. Future directions of research in lysophospholipid biology

Many of the remaining questions regarding Edg R mediation of LPL effects in complex tissues and organ systems may only be addressed definitively by genetic approaches, which modify levels of production of LPLs, LPL cellular transport systems and extracellular carrier proteins, and receptor expression and signaling. Several approaches have defined initial pharmacophores for each LPL mediator, but products of these efforts and several other potential synthetic agonists and antagonists lack sufficient bioavailability, specificity and

potency for meaningful in vivo or even in vitro studies. Such early results illustrate the difficulties of drug development when each physiological and pathological setting is characterized by a different mixture of bioactive LPLs and LPL receptors. Further development of LPL medicinal chemistry is expected to provide the necessary tools for assigning LPL signaling events to specific receptors. It is hoped also that emerging sets of Edg R-neutralizing antibodies will both represent early tools for initial cellular and animal studies, and facilitate standardization of assays needed for drug discovery.

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